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Cyanobacteria

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CYANOBACTERIA

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Meet the editor

Dr. Archana Tiwari is an associate professor at the Amity University, India. Her research interests include renewable sources of energy from microalgae and further utilizing the residual biomass for generation of value-added products, bioremediation through microalgae and microbial consortium, antioxidative enzymes and stress, and nutraceuticals from microalgae. She has been working on algal biotechnology for more than 15 years. Her research work has been published in many international journals with a high-impact factor. She has delivered talks as an invited speaker at many national and international conferences. She has authored many books and chapters with renowned publishing houses. She is also the recipient of 'Researcher of the Year Award' and 'Distinguished Scientist Award'.

Contents

Preface

Cyanobacteria are phototrophs with unmatched potentials and numerous applications in addition to the significant ecological roles they contribute in the environment. They have a great evolutionary history with a much eminent role and inhabit almost all geographic locations, including oceans, caves, ponds, lakes, soil, snow, and hot springs. The eutrophication in water bodies triggers the formation of blooms. Cyanotoxins are the toxins secreted by some cyanobacteria and are segregated on the basis of their mode of action into hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and irritant toxins. They can generate a variety of biofuels—hydrogen, ethanol, butanol, biodiesel, and methane. They find application in remediation of wastewater and heavy metal eradication in addition to their ability to reclaim soil, enhance the fertility of the soil, promote plant growth, act as biocontrol agents, and so on.

The bioactive compounds from cyanobacteria are quite enormous, and more than 800 compounds have been documented from marine cyanobacteria. In early 1500 BC, the therapeutic abilities of cyanobacteria were first acknowledged, and the genus *Nostoc* sp. was utilized in the treatment of diseases such as cancer, gout, and fistula. Later on, in 1900, Moore and Gerwick undertook extensive work on elucidating the role and application of cyanobacteria. Elaborative studies are untaken worldwide to unravel the untapped potential of cyanobacteria in the treatment of cancer, Alzheimer's disease, leishmaniasis, and many bacterial, fungal, and protozoan diseases. They are rich in antioxidative enzymes, and they find application in cosmeceuticals. The selective cyanobacterial compounds are in final clinical trials, which signify the conspicuous role they can play in the coming decade as substantial precursors of new-age drug molecules.

This book highlights the important aspects pertinent to the unique world of cyanobacteria. This book covers diverse topics and provides insights into the world of tiny microbial factories and their unraveled potentials.

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Section 1

The World of Cyanobacteria

Chapter 1

Cyanobacteria: The Wonderful Factories

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Additional information is available at the end of the chapter

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Abstract

Cyanobacteria are photosynthetic algae with outstanding endeavor to inhabit diverse habitats and are crowned with special metabolic acumen. Their morphological diversity is vivid and their ecological roles are magnificent and vital in nature ranging from nitrogen cycle to carbon dioxide mitigation. Their applications are now extensively explored and many novel compounds have been reported. The pigments, vitamins, lipids, proteins, polyketides, antioxidative enzymes, polysaccharides etc. derived from cyanobacteria are envisaged worldwide. Their diligent acumen makes them ideal tiny microbial factories for nutraceuticals, biofuels, cosmetics, pharmaceuticals, wastewater remediation and many more. Further investigations can aid in elucidating more cyanobacterial secondary metabolites and innovative approaches towards their wider applicability in plethora of avenues as sustainable reservoirs.

Keywords: cyanobacteria, novel compounds, nutraceuticals, secondary metabolites, wastewater remediation

1. The extraordinary photosynthetic microbes

Cyanobacteria are a distinctive class of extraordinary prokaryotes with photosynthetic capability loaded with immense potentials and diverse applications. Traditionally they were called '**blue-greens'**, as the first cyanobacteria reported were bluish-green in color. The fossils of cyanobacteria are 3.8 billion years old, among the oldest fossils currently known. They are pioneers of the major transformation that occurred in the due course of evolution contributing towards the aerobic metabolism as the photosynthetic machinery of angiosperms and photosynthetic eukaryotes are presumed to be developed from primitive cyanobacterium billions of years ago. Initially, Cyanobacteria was placed in the class of algae owing to their

morphology, photosynthetic pigments and oxygenic photosynthesis with photosystems (PS II and PS I) similar to the algae. Later on, Herdman et al. [1] reported that the genome size of cyanobacteria (1.6 × 109 to 8.6 × 109 Da) was similar to bacteria (1.0 to 3.6 × 109 Da) and it was advocated that cyanobacteria are more related to bacteria.

Cyanobacteria are microscopic in size but their colonies or mats are quite conspicuous. The habitats of cyanobacteria are quite diversified in terms of their unique adaptability to an array of climatic conditions ranging from glaciers, sea, and lake to deserts. They are one of the harbingers of the biological organism that evolved on earth perhaps after the first bacteria, billions of years ago long prior to mankind. They are dynamic organisms inhabiting the most extreme habitats on the planet and can be readily relocated to new avenues via air. The morphological dynamics include unicellular, filamentous and colonies. The cells of cyanobacteria are bigger in size compared to usual cells of bacteria. The nature of cell wall is peptidoglycan and is multi-layered with photosynthetic pigments in the outer part of protoplast. A covering of mucin is seen on the filament and no locomotion system has been reported, though some forms exhibit oscillatory motion [2].

They are photosynthetic in nature yet they are reported to inhabit marginally illuminated caves while on the other extreme end, they dwell well at salty marshes, exposed to high light intensity [3]. The photosynthetic machinery of cyanobacteria is armored with a myriad pigments- chlorophylls, carotenoids, and phycobiliproteins-phycoerythrin, phycocyanin and allophycocyanin [4, 5]. The pigment system enables the wide range of adaptations to the alterations in light intensities [6, 7]. An outstanding phenomenon called complementary chromatic adaptation is evident in cyanobacteria wherein they adapt to changes in the intensity of light due to the phycobiliprotein synthesis in response to the wavelength of light. The pigments also aid in protecting the cells from the detrimental effects of harmful radiations [5]. They inhabit virtually all major aquatic and terrestrial biome on the earth by virtue of their unique adaptability. The low water potential dwelling cyanobacteria resist the desiccations by adapting to the high salinity as seen in the ponds with hypersaline conditions [8]. The temperature range that permits the growth of cyanobacteria is quite large ranging from freezing to 40°C, though the optimum temperature lies in between 20 and 35°C, while the open ocean cyanobacteria are exposed to the temperature nearly 30°C [9]. The pH requirements of cyanobacteria generally range from neutral to alkaline, but they have also been reported to inhabit hot springs which are acidic in nature [10, 11]. The primary mode of nutrition in cyanobacteria is photosynthesis but in the hydrogen sulfide-rich environment, switching from oxygenic to anoxygenic photosynthesis is reported [12, 13] which is similar in nature to the bacterial type photosynthesis.

Cyanobacteria are very significant prokaryotes for the environment and plant growth. Though they are free-living organisms few live in symbiotic association with other eukaryotes and perform profound new roles essential for the ecosystem. They are the natural nitrogen fixings icons, which is quite essential for the entire biological system. The capability of converting atmospheric nitrogen into organic ammonia, nitrite or nitrate is called biological nitrogen fixation, though possessed by few organisms and essential for the growth of the plants. The talent of some cyanobacteria to bring about nitrogen fixation allows them to inhabit low nitrogen concentration ambiance, which is an added advantage in terms of survival and adaptability in the environment.

Cyanobacteria can serve as excellent sources of eco-friendly and renewable biofuels ranging from hydrogen to lipids as they are ideal microbes for production of biofuel based on their photosynthetic proficiency and their ability for genetic engineering [14]. They expedite in managing stress through an advanced antioxidative system. The antioxidative enzymes of cyanobacteria are efficient in protecting the cells from the damaging impact of free radicles generated through aerobic processes. The antioxidative enzymes Catalase, peroxidase, Superoxide dismutase, peroxiredoxins, Ascorbate Peroxidase etc. can be employed for commercial purposes and antioxidative therapy is the emerging concept in medical science [2]. The cyanobacterial pigments phycocyanin and phycoerythrin have great therapeutic value and are widely used in food, cosmetics and therapeutics in many parts of the globe. They are also reported to possess hepato-protective, antioxidants, anti-inflammatory and anti-aging activity along with positive effect in therapeutics of Alzheimer and cancer owing to their unique magnificent absorbance and fluorescence [15].

The Secondary metabolites from cyanobacteria are concomitant with hormonal, toxic, anti-neoplastic and antimicrobial effects [16, 17]. The range of antimicrobial efficacy ranges from the plethora of microorganisms, prokaryotes and eukaryotes. They are great reservoirs of diverse varieties of secondary metabolites. The toxins secreted by cyanobacteria are diverse from hepatotoxins to lipopolysaccharides and are extensively research worldwide [18].

Cyanobacteria have multifaceted applications in the modern biotechnological and pharmaceutical arena. They are also broadly used in the treatment of wastewater, in aquaculture as fish feed, food, fertilizers, and various secondary metabolites like toxins, exopolysaccharides, enzymes, vitamins, and nutraceuticals [19]. Cyanobacteria also find applications as UV-absorbing amalgams, bioplastics (polyhydroxyalkanoates, PHAs) and coating material etc. and have tremendous bioindustrial potential [2]. These wonderful tiny factories are yet to be explored so that they can be exploited for a sustainable world, a better tomorrow. This book highlights the significant studies on Cyanobacteria by authors from the parts of the world envisaging the characteristics features and applicability of these special prokaryotes.

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Section 2

Microscopic Dimensions

Chapter 2

Fluorescence Microscopic Spectroscopy for Investigation and Monitoring of Biological Diversity and Physiological State of Cyanobacterial Cultures

Natalia Grigoryeva and Ludmila Chistyakova

Additional information is available at the end of the chapter

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Abstract

In this chapter, a novel technique for investigation of natural and laboratory cyanobacterial cultures is presented. The technique is based on a strict relation between the intrinsic single-cell fluorescence emission spectra of cyanobacteria and the physiological state of the whole culture. It will be shown else that the single-cell fluorescence spectra for different species are steady enough to conduct a taxonomic analysis of cyanobacterial cultures based on a common statistical data evaluation among the parameters extracted from a set of such spectra. Several examples are given to illustrate the power and simplicity of a new technique, which can become a promising tool for automation of production in the cyanobacterial biotechnology, as well as give a valuable contribution to the development of innovative approaches in environmental monitoring of harmful algal blooms.

Keywords: cyanobacteria, confocal laser scanning microscopy, single-cell fluorescence spectrum, biological diversity, physiological state, environmental monitoring, biotechnology, harmful algal blooms

1. Introduction

Cyanobacteria have gained huge attention in recent years because of their potential application in biotechnology [1–5]. For example, cyanobacteria are considered as a rich source of biologically active compounds with antiviral, antibacterial, antifungal and anticancer activities. Several strains of cyanobacteria were found to accumulate polyhydroxyalkanoates, which can be used as a substitute for nonbiodegradable petrochemical-based plastics. Recent studies showed that oil-polluted sites are rich in cyanobacterial consortia capable of degrading

oil components. Cyanobacterial hydrogen has been considered as a very promising source of alternative energy and has now been made commercially available. Cyanobacteria are also used in aquaculture, wastewater treatment, food, fertilizers, agriculture, production of secondary metabolites including exopolysaccharides, vitamins, toxins, enzymes and pharmaceuticals. In addition, the ecological aspect of the harmful bloom monitoring and control makes an important contribution in this rising interest to cyanobacterial problem.

In the last few years, the investigation of taxonomy, physiology, morphology and genetics of cyanobacteria attracts a considerable attention. A vast amount of different techniques were elaborated to achieve, nowadays, an insight into the physiological processes that rules cyanobacterial life and their genetic background. Large-scale industrial production of the cyanobacterial products requires optimization and more detailed control of incubation conditions in order to increase productivity. Future research will be focus on isolating and study of new cyanobacterial strains and the improvement of different treatments that will support or inhibit their growth.

In this chapter, the novel and most powerful part of the optical spectroscopy, which could make a considerable contribution to the future investigations—a confocal microscopic spectroscopy—will be presented and illustrated by several examples of application. It should be noted, that due to space limitations, only few citations could be incorporated in this chapter. They represent a limited selection from a large amount of works and should be used as a source for further references.

As it was pointed out earlier, there are two opposite aspects in the cyanobacterial problem: first, to prevent ecological hazards, that is, toxic cyanobacterial blooms, and, second, to improve industrial incubation of cyanobacteria, involved in such important applications, as food and fuel production. The former deals with the study and treatment of natural samples and includes the investigation of biological diversity, monitoring of physiological state of natural communities, record and analyze the results of external actions and changings in environmental conditions. In many situations, taxonomic phytoplankton composition is of crucial importance when toxic or other harmful substances might be produced by cyanobacteria. The latter aspect concerns the registration and control of the optimal physiological state and viability of the laboratory or industrial culture in specified conditions. Thus, there are two main problems that are involved in all areas of application mentioned above and meet some obstacles while using conventional methods of investigation. They are: a correct classification and discrimination of present and new cyanobacterial strains, and monitoring of physiological state of cyanobacterial cells in natural communities and in laboratory cultures during industrial incubation.

All these different tasks deal with a big data processing and possibility of process automation are quite desirable. Confocal microscopic spectroscopy gives a unique opportunity for direct automation of all these processes or, otherwise, indirect application of the results of the detailed single-cell spectroscopic investigations for implementation in innovative devices or technique.

The taxonomic composition of cyanobacterial communities is of interest in water-quality field and ecology, where the effect of nutrient pollution on coastal and freshwater resources should be controlled [6, 7], as well as in industrial biomass production, where the additional undesirable strains may appear during the long cultivation process. In nature, the composition of phytoplankton communities can be highly variable in space and time [8, 9]. Characterization of the

community composition, therefore, requires frequent, high-resolution sampling. Historically, community characterization has been done by chemical preservation of samples and analysis by bright-field or epifluorescence microscopy. Although optical microscopy allows direct measurements of cell size and identification to species level it is laborious and time-consuming, limiting the number of samples that can be analyzed in a day. Minor variations in the composition of phytoplankton are consequently not revealed when using optical microscopy technique. More recently, *in situ* flow cytometric instruments capable of automated characterization of phytoplankton communities have been developed [10, 11]. These instruments have excellent resolution over a wide range of cell sizes but have a great disadvantage of high requirements for sample preparation and no possibility of cell-viability control. Alternative methods that are based on differences in accessory pigments among phytoplankton taxonomic groups [12] such as chemotaxonomic and spectrofluorometric methods have been proposed and included. First requires the high pressure liquid chromatography (HPLC) analysis of pigment contents [13–15]. The introduction of pigment analyses by HPLC facilitated easy and accurate separation, identification, and quantification of phytoplankton pigments. The large number of samples that can be processed by HPLC allows a more thorough examination. However, it does not allow for high-resolution data acquisition and again give no information about physiological state of single cells. And the last, spectrofluorometric method, although enables low-cost, rapid measurements, but till now deals with fluorescence-based chlorophyll *a* (*Chla*) quantification methods, that were proposed by several authors in the early 1960s and were applied either *in vitro* or *in vivo* to continuous measurements of algae and higher plants [16–22]. Unfortunately, these methods cannot be directly applied to cyanobacteria and usually give incorrect results.

Recently, attempts to conduct the discrimination among microalga on the base of absorption or fluorescence spectra were reported [19, 21, 23–26]. Most of them use only absorption spectra. Absorption spectrum includes the information only about the chemical structure of photosynthetic cells, so it results in a rough discrimination of big classes of phytoplankton: diatoms, dinoflagellates, prymnesiophytes, euglenophytes, prasinophytes, raphidophytes, cryptophytes, chlorophytes, chrysophytes, and cyanobacteria. However, among the species of one class, for example, cyanobacteria, the chemical characteristics are quite similar, except several cases when phycoerythrin occurs in phycobilisome in addition to phycocyanin as an accessory pigment. In the last case, the only differentiation can be made among two big groups: the species containing phycoerythrin and those who lack it. All other differences are so small that cannot be used for further differentiation of cyanobacterial species and strains, so more precise classification, that is, among cyanobacterial species and strains, is impossible using only the absorption spectra.

Opposite to the absorption spectra, the *in-vivo* fluorescence spectra are much more informative. Fluorescence detection is undoubtedly a powerful tool owing to the existence of natural fluorescence from phycobilins and chlorophylls. It is a highly sensitive, nearly instantaneous, noninvasive way to study various components and processes *in situ* and *in vivo*. Although the fluorescence spectra contain the information only about photosynthetic apparatus of different algal groups, they include the information about the chemical structure of light harvesting complex (LHC) and accessory pigment-proteins, as well as about the character of links between pigment-protein complexes and the efficiency of energy transfer in the light harvesting process. When compared with absorption, fluorescence is affected by the excitation wavelength and energy. Thus, the use of different excitation wavelengths can provide more detailed information for the study of single-cell composition.

Fluorescence spectra have been used to classify phytoplankton populations since the approximately early 1970s [20]. However, because of the generally low number of available excitation wavelengths in the conventional devices the rate of species discrimination was relatively low. Researches again can separate only algal groups that differ greatly in pigmentation and, therefore, in fluorescence spectra (e.g., cryptophytes, chlorophytes and cyanobacteria), but cannot separate groups that are more similarly pigmented (e.g., among cyanobacterial species) [22, 27]. Discrimination between similarly pigmented taxa or even between species within a taxon requires high-resolution spectra and the use of a set of excitation wavelengths to reveal small peculiarities in configuration and functioning of light harvesting system. The rigorous discrimination is possible if the inter-species differences are greater than those within a species. These requirements can be fulfilled only when for each species a set of single-cell fluorescence spectra, excited by a number of wavelengths, are obtained and analyzed. The possible contribution of environmental adaptation effects to the resulting fluorescence spectra can be minimized by an accurate definition of the corresponding spectral regions in the spectra under consideration.

The problem of registration and control of the physiological state of single cells in natural communities and the viability of cultures during incubation is a primary task in both ecological and biotechnological fields of application of cyanobacteria. This problem is more complex than the species discrimination because it deals with very weak variations in chemical and optical characteristics of single cells and a culture as a whole. Despite the importance of this problem in vast amount of tasks, the methods for studying and monitoring of physiological state of cyanobacteria are still based mainly on traditional optical methods of registration, as well as on the analysis of fixed or dissociated samples [28–31]. These approaches do not allow one to register small changes in the physiological state of cyanobacterial cells, which are extremely important during weak external treatments or environmental changes. Usually, this study is reduced to a manual counting of the total number of cells in the experimental samples and determining the total volume of chlorophyll [32–34].

The last method is very effective for algae and higher plants, where chlorophyll is a main pigment, determining the viability. It quickly disintegrates in dead cells and therefore can really serve as an indicator of the viability of single plant. In cyanobacteria, the main pigments responsible for photosynthetic activity are phycobilins, and chlorophyll does not disintegrate for a long time in dead cells, even after their disruption. Therefore, the methods associated with the analysis of the chlorophyll fraction do not give satisfactory results in the study of cyanobacterial cultures. The analysis of the light fraction of water-soluble phycobilins may give some additional information, but it is also ambiguous, since the pigment-protein complexes in the disrupted cells degrade rapidly and carry no information about the initial viability of living cells. With such approach, weak changes in the physiological state of cyanobacterial cells cannot be detected, since the results of the experimental treatment are more influenced by the used methods of investigation than the directional external action, which is studied.

The only method that seems to be appropriate for physiological state investigation and can diverse live and dead cells is the delayed fluorescence (DF) technique. Delayed fluorescence is the long-term emission of light from cells triggered by illumination [27, 35, 36]. It has the same emission spectrum as chlorophyll *a* fluorescence, but occurs with a time delay (from milliseconds to minutes) [37]. The major advantage of DF is that it is emitted only from cells

that are photosynthetically active, that is, alive. Thus, additional signals from dead cell debris do not interfere with the measurements. Long-term DF emission also prevents interference problems with fluorescent backgrounds in natural samples [38]. Furthermore, DF can measure nano- and pico-plankton, which may be lost during filtration or may be unaccounted in direct microscopic analysis. However, by means of DF, only the ensemble spectra of the whole culture can be measured and the physiological state of individual cells is unavailable as before. Thus, a new precise, nondestructive and sensitive method for registration of weak reversible and irreversible changes in the physiological state of cyanobacterial cells should be elaborated.

Historically, since the 1960s, investigators have noted that changes in the physiological state of cyanobacterial cells occurring when it is damaged are reflected in the corresponding changes in the intrinsic fluorescence spectrum [39]. Several studies have shown that a decrease in the pigment fluorescence is associated with a decrease in the enzymatic activity of the cell and an increase in the permeability of the cell membrane, which can be used as an indicator of aging for cyanobacterial species [31]. However, this fact has not yet been widely used to assess the viability of individual cells of cyanobacteria and cultures.

To date, there is no doubt that the *in vivo* analysis of fluorescence parameters of light-harvesting complexes is a powerful tool for studying the effect of a wide variety of environmental factors on photosynthetic organisms. The intensity of fluorescence emitted by single photosynthetic cells *in vivo* depends only on the structure and operational effectiveness of photosynthetic apparatus, reflecting the individual characteristic of cyanobacterial strain and in-time physiological state of the cells under consideration. The environmental changes cause the changes in bioenergetic processes occurring in cyanobacterial cells; they significantly affect the kinetics parameters and spectral features of the intrinsic fluorescence of photosynthetic apparatus. Thus, the intrinsic fluorescence spectra of a particular type of cyanobacteria, the so-called "fluorescent fingerprints," can be used to identify photosynthetic pigments and to determine the viability of individual cells, as well as for preliminary taxonomic analysis of full-scale samples [40, 41]. These "fluorescent fingerprints" can be easily obtained by the routine lambda-scanning at most of confocal laser scanning microscopes.

We present a novel technique based on a strict relation of the physiological state of cyanobacterial cells and their genera affiliation with the intensity and shape of the intrinsic single-cell fluorescence spectra, obtained by means of confocal microscopic spectroscopy. The nondestructive spectral analysis conducting *in vivo* at a cellular level allows to obtain more complete information about special features of individual cyanobacterial cells and supports the registration of very weak variations in their physiological state. The application of this technique for automation of control processes gives an additional opportunity to rise an effectiveness of production in biotechnology and brings in a valuable contribution to the development of innovative approaches in environmental monitoring.

In this chapter, two main application problems are investigated by means of fluorescent microscopic spectroscopy. Firstly, how the composition of photosynthetic pigments affects the shape of *in-vivo* single-cell fluorescence spectra and, secondly, how the differences in the fluorescence response of cyanobacterial cells may be used for investigation of their physiological state and biological diversity.

2. Fluorescence microscopic spectroscopy

Modern fluorescence microscopic spectroscopy (FMS) or confocal laser scanning microscopic spectroscopy provides a unique opportunity to obtain high-resolution images and intrinsic fluorescence emission spectra from single cyanobacterial cells [42–45]. Moreover, using spectral unmixing, the fluorescence of individual spectral components can be resolved, and their relative intensities can be calculated [46–48]. Unfortunately, most of the researches use confocal laser scanning microscopy only for imaging [49–54]. In this part, the attention is paid mostly to the spectroscopic studies by means of CLSM, and we will give some guidelines on methods of investigation and sample preparation.

It is well known that measurement and analysis of fluorescence is one of the most powerful ways to probe photosynthetic systems because it reports on the energy transfer and trapping. This fluorescence originates from excited states that were lost before photochemistry took place. It usually represents a small fraction of the excited state decay in a functional photosynthetic complex. Nevertheless, this small fraction can be easily detected by CLSM. With the confocal fluorescence microscopy, a very small excitation and detection areas can be investigated, so that single cells under non-damage conditions can be studied *in vivo*. Although, the pigment structure of different cyanobacterial strains has been intensively investigated, the variations of *in-vivo* operation of photosynthetic apparatus for different cyanobacterial species have not been analyzed yet. We suppose that the best way to investigate the operation of photosynthetic system *in vivo* is a single-cell fluorescence spectroscopy. Single-cell detection can provide the information on small peculiarities that is regularly buried in normal ensemble average experiments. This is thus a good way to study the time evolution process and spectroscopic properties of individual cells. Both steady-state and time-resolved fluorescence measurements can be used for probing the organization and functioning of photosynthetic systems by means of CLSM.

2.1. CLSM parameters

In the presented investigation, Leica TCS-SP5 was used for spectral CLSM of living cyanobacterial cells. Fluorescence emission spectra of the intact cells were measured at eight excitation wavelengths corresponding to all available laser lines. The excitation wavelengths are: 458, 476, 488, 496, and 514 nm—the lines of Ar laser, 405 nm is the line of diode UV laser and 543 and 633 nm are the lines of HeNe laser. In the experiments, presented below in this chapter, laser power settings were as follows: 29% of Ar laser power was reflected onto sample with acousto-optical tunable filter (AOTF) and further power percentage for its laser lines was: 30% of 458 nm laser-line and 10% for all other lines. 405 nm line of diode UV laser was reflected onto sample with 3%, HeNe laser lines 543 and 633 nm were reflected with 10 and 2%, respectively. An acousto-optical beam splitter (AOBS) was used to transmit sample fluorescence to detector. Emission spectra between 520 and 785 nm were recorded using the lambda scan function of the "Leica Confocal Software" by sequentially acquiring a series ("stack") of 38–45 images, each with a 6-nm fluorescence detection bandwidth and with 6 nm wavelength step. For obtaining fluorescence-intensity information images of 512 × 512 pixels were collected with a 63× Glycerol immersion lens (glycerol 80% H_2O) with a numeric aperture of 1.3 (objective HCX PL APO 63.0 × 1.30 GLYC 37°C UV) and with additional digital zoom factor

5–9. 1 pixel corresponds to 53.5 × 53.5 nm. The photomultiplier (PMT) voltages were used in range from 900 to 1100 V. The fluorescence emission images were accompanied with the transmission images (in the parallel channel), collected by a transmission detector with the photomultiplier voltages ranged from 300 to 500 V. For better signal yield, lambda scans were performed with "low speed" setting (400 Hz) in bidirectional scan mode and with a pinhole setting of 1 Airy unit (the inner light circle of the diffraction pattern of a point light source, corresponds to a diameter of 102.9 μm with the lens used (see [46]). Regions of interest (ROIs) representing single cells or subcellular regions were used to calculate fluorescence spectra.

2.2. Prevent photobleaching

In CLSM applications, the laser light density in the focus point is high. But, generally, it is difficult to compare the excitation energies used in CLSM with those from methods developed to measure photosynthetic parameters. In CLSM, light is deposited in short "dwell times" during the laser scanning process. Dwell time and the intervals between the illuminations may influence photo-damage and saturation of photosynthesis. Thus, since most chromophores bleach under the high laser excitation energies, a bleach-test should be performed [43]. It was shown experimentally that especially phycoerythrin (PE) and phycocyanin (PC), as an accessory pigments, were very sensitive to photo-bleaching, while the fluorescence of Chlorophyll *a* (Chl *a*) and allophycocyanin (APC) remained stable in the intact cells [43]. During the detection, the fluorescence of the main accessory pigments for each cyanobacterial strain should be controlled and the changes in their fluorescence should not exceed 10–20%. The power of individual laser lines should be chosen according to the photodamage they cause. In our experiments, the repeated spectra were obtained under selected excitation power at a fixed point in a cell to check whether the excitation would affect the cells. It was shown that at the above chosen excitation energies, the fluorescence spectra did not vary within the experimental error during 10–15 records. When excitation energy was increased, both the height and the center of the bands varied enormously with time because of photodamage or structure-breakdown in photosynthetic systems. In the experiments, where several laser lines were involved for the investigation, the first spectrum was recorded again at the end of each series to control the initial state of the cell. To compare different cells in one physiological state, the fluorescence spectra were taken from the cells of one strain cultured at different days and it was established that the variations in spectrum shape and intensity are not considerable. To visualize differences between strains with higher spectral and spatial resolution, lambda scans were performed with 6 nm bandwidth and with 6 nm steps. As far as the fluorescence intensities depend on the excitation energy (which varies for different laser lines), sensitivity setting of the photomultiplier, and the distance from the sample, all spectra were usually normalized to their maximum and only qualitative analysis was performed.

2.3. A set of excitation wavelengths should be used during the investigation

It is well known that phycobilisome contains several kinds of biliproteins, and its absorption and fluorescence spectra reflects the contribution of each. On the other hand, as a result of energy transfer among the tightly coupled biliproteins in the phycobilisome, fluorescence of the intact living cyanobacterial cells is originated from the efficiency of the energy transfer between these components and each transfer step appears in the spectrum shape as peak or

shoulder (**Figure 1**). Moreover, depending on the excitation wavelength, the room temperature fluorescence emission spectrum of intact cyanobacterial cells exhibits various extents of contribution of phycobilisome emission to the spectrum. If one exclusively excites Chl *a*, using a 458 nm line of an Ar laser, the emission spectrum by cyanobacterial cells shows no appreciable emission of PC or APC. In cyanobacteria, the 458 nm excitation is preferentially absorbed by photosystem I (PSI) that contains more Chl *a* than by photosystem II (PSII) and is stoichiometrically more abundant than PSII. However, because reaction center of PSI turns over faster than the PSII, it has lower fluorescence intensity than the PSII antenna. This is indicated by PSI emission band at 715 nm which is much weaker than the PSII emission band at 682 nm. The excitation by intermediate (blue and green) wavelengths (405, 488, and 496 nm) reveals fluorescent maxima of all photosynthetic pigments, as the light in this range is absorbed by all pigment-protein complexes almost in equal portions and fluorescence emits by all steps of energy transfer chain (**Figure 1**). The direct excitation of cells in the PC absorption region at 514 and 543 nm, results in emission spectrum with two main peaks at 580 and 656 nm, which are due to PE, PC and APC emission and for species that lack PE the emission accumulates

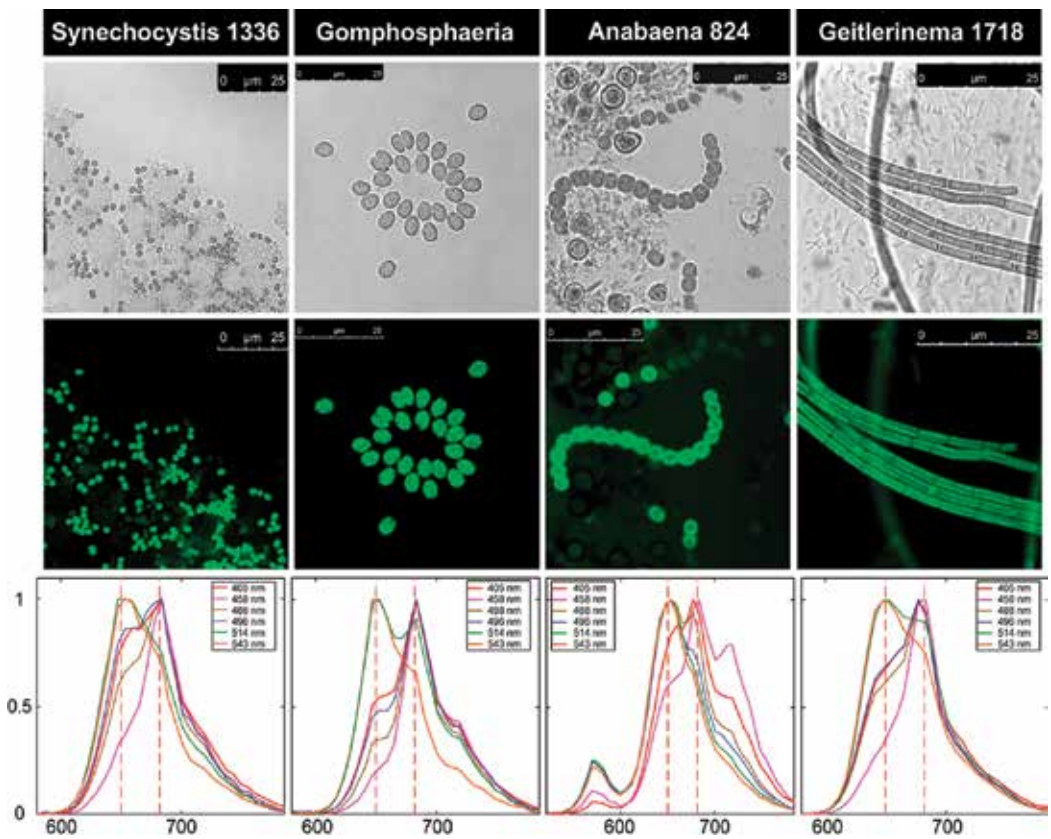

Figure 1. The examples of CLSM images and normalized single-cell fluorescence spectra for four cyanobacterial strains. The white bar corresponds to 25 μm. Corresponding excitation laser lines are indicated in plot legend. Dashed lines indicate fluorescence wavelengths of PC and Chl *a* fluorescence at 656 and 682 nm, respectively.

mostly near 656 nm. The spectra of the 633 nm excitation (not shown here) directly give a prominent emission band at 656 nm, that originates from PC, omitting band at 580 nm, which cannot be excited by 633 nm, even for species that have PE. Other small emission bands, corresponding to fine pigment structure of antenna complex, are not resolved in the room temperature investigation. Comparative analysis of the series of fluorescence spectra for different cyanobacterial species and strains reveals visible variations in their shape (**Figure 1**). If the fluorescence spectra were taken from alive cells in normal physiological state, which are cultured in the same growth environmental conditions, then the interspecies variations in pigment/chl *a* ratios are more pronounced than variations within the individual species. Species/strains differentiation could be carried out on the base of fluorescence analysis.

2.4. Investigation of physiological state of single cell

As it was pointed out, the single-cell fluorescence spectra depend not only on the selected strain, but also on the physiological state of the chosen cell. CLSM is the only method that can accurately diverse alive and semi-dead cells (**Figure 2**), and compared to other direct fluorescence measurements, records the fluorescence emission spectra from only active pigments in alive cells and does not acquire information from dissolved pigments, organic substances, and debris around. Considerable differences in shape and intensity of the fluorescence emission spectrum of cyanobacterial cells within one strain in normal and depressed physiological states allow to estimate the viability of the whole culture relying not only on the visual methods, but also on the accurate spectral analysis. In **Figure 2** (right panel), three typical fluorescence spectra for three different physiological states of one microcystis cell are presented. Spectrum I corresponds to the alive cell, spectrum II is the spectrum of cell in the depressed physiological state, and spectrum III is a spectrum of the dead cell. All spectra were excited by 488 nm laser line. Here the normalization to maximum intensity was not made, so that to illustrate the considerable difference in intensities between normal and depressed cells. On the left panel, corresponding transmission and fluorescent images of the cell under

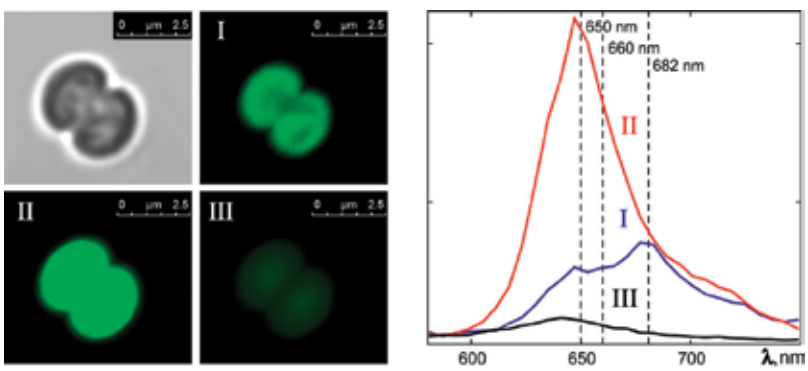

Figure 2. CLSM images and single-cell fluorescence spectra (in relative units) for cyanobacterial strain *Microcystis CALU 398*, obtained at three different physiological state of the same cell: I—spectrum of the alive cell, II—spectrum of the cell in depressed physiological state, and III—spectrum of the dead cell. The white bar corresponds to 2.5 μm. Dashed lines indicates fluorescence wavelengths of APC, PC and Chl *a* at 650, 660 and 682 nm, respectively.

investigation are shown. Analyzing the shape of the ensemble average fluorescence spectrum and counting the relative number of alive and semi-dead cells the conclusion about the viability of the whole culture at given developmental stage could be made.

2.5. Sample preparation

Special attention should be paid to the sample preparation, as well as we work with alive objects. Coverslip should be pressed very carefully to prevent any glass slide, which can cause cell damage. On the other hand, one should keep in mind that cyanobacteria can move and glide; so to fix the object, the coverslip should be pressed hard enough to prevent any motility of the investigated object, which sometimes have a diameter near 1 μm (e.g., microcystis and synechocystis cells).

2.6. Spectral unmixing

There is another very powerful tool implemented in CLSM—spectral unmixing. Unfortunately, in living cyanobacterial cells, it meets some difficulties. The authors of [43] pointed out that a lot of problems arise during the spectral unmixing procedure, which is based on the spectra of isolated phycobiliproteins. These problems are caused by the fact that the light absorption and emission properties of isolated phycobiliproteins are rather different from those of the intact phycobilisomes in the living cyanobacterial cells. In living cells, the spectral properties of pigments from certain organisms may differ crucially from the properties of the dissolved ones, for example, spectra of the components can vary in peak widths and may be shifted in wavelength due to different pigment-protein and linker connections. Thus, the analysis based on the initial fluorescence spectrum without any decomposition is preferable for living cells.

3. Examples of fluorescent microscopic spectroscopy application

The detailed description of the morphology, structure, chemical and optical properties of light-harvesting complex of cyanobacteria, phycobilisomes and phycobilins can be found in numerous publications [55–72]. On the other hand, the fluorescence properties of the intact living cyanobacterial cells differ drastically from the properties of the detached phycobilisomes and its components and originate from the efficiency of the energy transfer between all components of the energy transfer chain included the final step, the delivery to PSII or PSI. Each transfer step result in the spectrum shape as a peak or shoulder. Moreover, fluorescence of photosynthetic pigments in the intact cells is affected by physicochemical and physiological processes that occur within and across the thylakoid membranes. Here we demonstrate on several examples how these peculiarities can be used for investigation of physiological state and biological diversity of cyanobacteria.

The correct identification of cyanobacterial cultures and estimation of their physiological state are quite important in the environmental monitoring and industrial applications. The ability to detect small variations in the physiological state of cyanobacterial culture under weak external treatments is quite desirable in both field and laboratory experiments. The fluorescent CLSM technique is a very powerful tool that can support any on-line field, laboratory

and technological study. In addition, the results of single-cell spectroscopic analysis are much more suitable for further statistical and analytical calculations then the conventional optical methods of investigations. In this chapter, we present several examples of practical application of the described CLSM technique.

3.1. Monitoring of physiological state of single cyanobacterial cell and a whole culture

Since the first broad-scale spectroscopic investigations, the authors of many articles note the dependence of the intrinsic fluorescence spectra of cyanobacteria on the developmental stage of the culture and physiological state of single cells. It is well-known that the light-harvesting and energy-transfer capacities of phycobilisomes can react to the environmental changes, as well as to the changes in physiological state of the living cells induced by stress conditions [39, 64, 73–75]. However, this effect has not yet been widely used to assess the viability of the culture. Several authors pointed out that, although a single-cell fluorescence spectra for the diverse physiological states differ significantly, the physiological state of the given cell cannot be estimated correctly because of the absence of a full set of reference spectra [40, 76, 77]. Moreover, the authors of [40, 76, 77] pointed out that while comparing spectra of individual cells and the results of the ensemble average experiments at a culture as a whole (so-called integral spectra), a significant difference was observed. Obviously, this difference is owing to a wide diversity of single-cell physiological states in the bulk growing culture, which in sum gives different integral fluorescence spectra for a specified strain at different developmental stages because of the variations in cell's proportions. This, of course, should be taken into account. On the other hand, the whole culture in addition to a set of single living cyanobacterial cells consists of metabolites, dissolved pigments, other organic substances and cellular debris. All these substances form undesirable and unpredictable fluorescent background in volume samples.

Actually, the intensity of fluorescence emitted by single photosynthetic cell *in vivo* depends only on the structure and operational effectiveness of photosynthetic apparatus, tracing in-time physiological state of the cyanobacterial cell. Thus, the fluorescence emission can be used effectively to monitor various physiological processes. **Figure 3** illustrates the temporal changes of in-vivo fluorescence spectrum taking place in one living cell of cyanobacteria strain *Synechocystis CALU 1336* under light and heat stress. On the other hand, this timeline set of fluorescence emission spectra illustrates all stages of cyanobacterial cell degradation, that is, all possible physiological states. It is obvious that during the evolution of the culture and aging of each cell all this stages will be presented in the natural samples simultaneously.

Several newer publications [76–80] clearly demonstrate that the variations in the fluorescence shape and intensity of living cells, presented in **Figure 3**, indicate the consequent degradation in the light harvesting chain (antenna complex—reaction center) and following dissociation of the detached antenna complex. It can be seen that in the alive cell, the chlorophyll *a* fluorescence prevails over the fluorescence of the pigment-protein complexes of phycobilisome. While the single-cell physiological state changes for the worse, the photosynthetic apparatus shows instability in operation, that is, the most part of the absorbed energy emits as fluorescence at the early stages of light harvesting. At the last stages, the changes in fluorescent spectrum, shown in **Figure 3**, are the same as it was demonstrated in the works [80–82], where

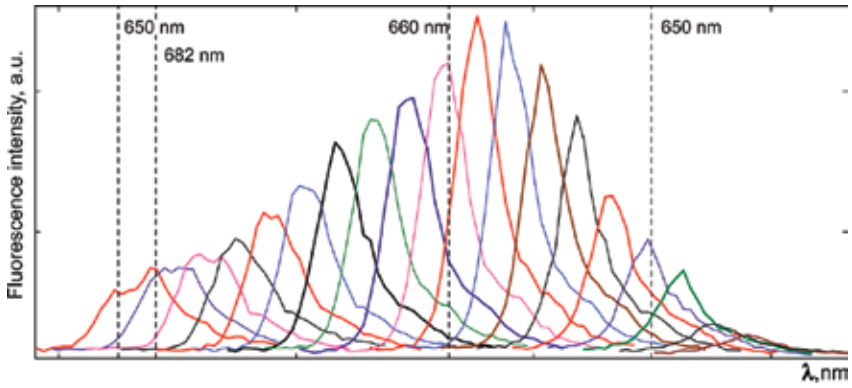

Figure 3. Time degradation of living cell of cyanobacterial strain *Synechocystis CALU 1336* under light and heat stress. Spectra were recorded at the excitation wavelength 488 nm and with the time step 2 min. Spectra are shifted along *x*-axis relative to each other for convenience of observation.

the dissociation of phycobilisomes was investigated. Thus, the estimation of the viability of single living cell and the whole culture is possible via investigation of the changes in fluorescence emission spectra.

Here we present a novel technique, recently elaborated by authors of this chapter, which is based on a strict relation between the shape and intensity of a single-cell fluorescence spectra of cyanobacteria and the physiological state of this cell. This technique is a direct extension of the previously elaborated visual methods of the estimation of the physiological state of cyanobacterial cells by the color of their fluorescence conducted by means of conventional fluorescent microscopy [39]. In contrast to the latter, which is very subjective, a new technique provides a detailed spectroscopic information and variations in "color" of the fluorescence can be measured in nanometers of light wavelength. A general character of the presented technique makes it possible to use it for investigation of any species of cyanobacteria, regardless of their habitat or cultivation conditions. Also the influence of scattering particles and preillumination effects, which are very important in the ordinary fluorescent methods, are absent in single-cell microscopic spectroscopy technique. Moreover, the use of the novel methods of microscopic spectroscopy allows to estimate the viability of colonies of noncultivated cyanobacterial species in natural samples according to the physiological state of individual cells. This fact can considerably facilitate the work with small concentrations of objects under consideration in environmental probes.

According to the proposed technique, the research process can be divided into three steps:

Step 1. Obtaining of the complete set of single-cell fluorescence spectra for given cyanobacterial strain in different physiological states by recording a series of spectra during single-cell degradation by means of CLSM at a certain excitation frequencies (e.g., at 458, 488 and 514 nm), as it was previously shown for 488 nm laser line in **Figure 3**. These sets will serve as reference spectra while determining the rate of cell degradation. At the same time, the most informative spectra for further investigation should be selected, which reflects the physiological state of the cells of a given strain.

Step 2. Recording of several sets of fluorescence spectra for single cells from the tested sample, which belongs to different physiological states, according to obtained reference spectra for

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this given strain. For accuracy of further estimation of the viability of the whole culture, these spectra should be recorded at several excitation wavelengths for each cell (in our investigations it was set of eight laser lines) and then each one should be averaged through a number of cells. The fluorescence spectra of a single cell excited by one laser line is not enough to have a complete information about deviations in photosynthetic process and pigment composition.

Step 3. Direct study of the tested sample in order to determine the viability of investigated culture or colony. A representative random sampling is used for the cells from a given strain and single-cell fluorescence spectra excited by chosen excitation wavelengths are recorded. At this stage, two variants of investigation are possible according to the final purpose.

If the aim is to estimate the viability of a specified culture or colony, one can directly count the rate of normal and depressed cells by their fluorescence emission characteristics and make a decision about the viability of the whole culture. In different periods of culture development, the percentage of living and depressed cells changes significantly. If the percentage of alive cell spectra prevails in the sample, then the culture is supposed enough viable. If the sample has more spectra of sick and dead cells, then the culture is weak and incapable of active reproduction. Alternatively, one can make a linear combination from several spectra, belonging to different physiological states, and compare this result with the integral spectra of the whole culture, obtained via conventional fluorimeter. The fitting coefficients in this linear combination will show the rate of the viability of the considered culture. The second way is less accurate, due to the undesirable fluorescent background in the whole culture mentioned above, and can be applied only for the fresh culture at the early growth phase.

If the main purpose of the investigation is to reveal the influence of weak external actions or environmental changes on the physiological state of single cells from the tested sample, the comparative analysis of single-cell fluorescence spectra from the control and treated culture should be carried out. In this case the investigation of the cells in normal (good) physiological state is enough. If any changes in the shape or intensity of single-cell fluorescence spectra are registered compared to the reference sampling, thus the influence takes place. Independently to the form and the sign of this changes, one can fix the result of the external action; however, it is impossible to consider the origin of this result. For the latter, the additional precise investigation by means of other physical or chemical methods has to be done.

Let us illustrate the effectiveness of this technique on a concrete example of the estimation of the viability of the culture of cyanobacterial strain *Synechocystis CALU* 1336 (from CALU collection), provided by of the Core facility Center for Culture Collection of Microorganisms of Saint-Petersburg State University.

The experiment was carried out in the following way. After passing through steps 1 and 2, for the considered culture the spectra of the individual cells in the normal and depressed physiological states were recorded by means of CLSM at eight excitation wavelengths and averaged for each state (**Figure 4a–c**). Usually, for the culture in exponential growing phase, the range of physiological states includes almost all developmental stages presented in **Figure 3**, but only two main single-cell physiological states—"healthy cells" and "cells in strongly depressed state"—can be selected for further calculations, as it was previously shown in **Figure 2** (states I and II, respectively). These two basic sets of spectra for the main physiological states are shown in **Figure 4a–c**. Blue lines show healthy cell in good physiological state, red lines correspond to the strongly depressed cells. For the clarity of further narration, only three of the

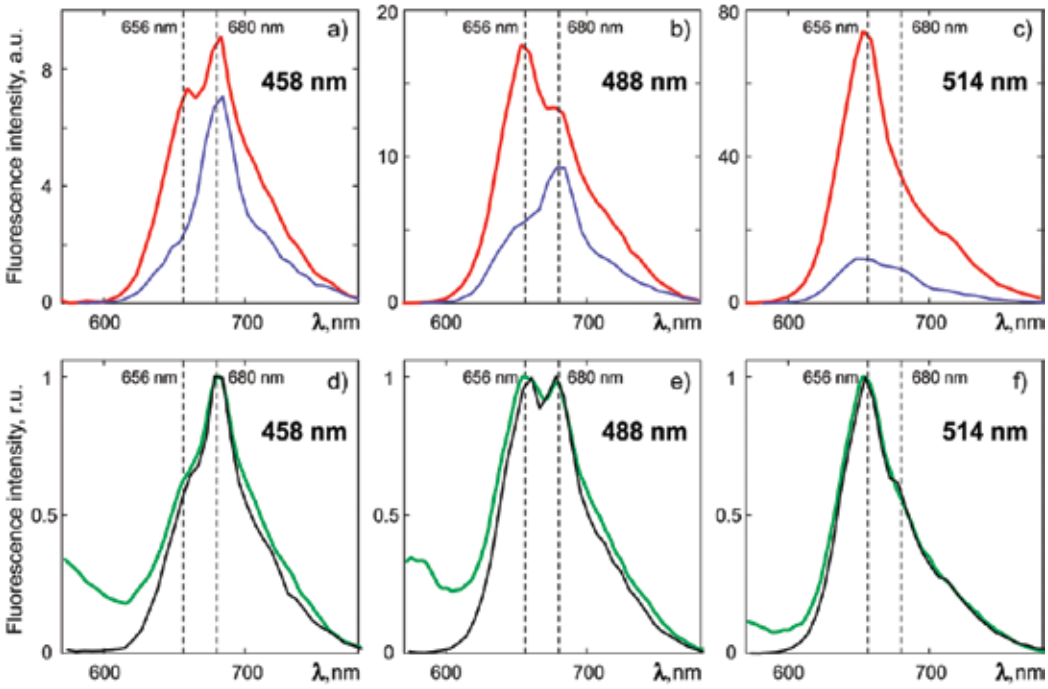

Figure 4. The illustration to the process of the culture viability estimation. (a–c) Averaged SFS for cyanobacterial strain Synechocystis CALU 1336, excited by three laser lines 458, 488, and 514 nm. *Blue* lines show spectra of alive cells, *red* lines are for the cells in the depressed physiological state. (d–f) Fitted and integral normalized spectra for the whole culture under consideration. *Black* lines show a linear combination of CLSM spectra of alive and depressed cells summed in ratio 2:1. *Green* lines show the integral spectra of the whole culture obtained using Cary Eclipse fluorimeter (Varian Cary) at corresponding excitation wavelengths. At all plots dashed lines indicate the fluorescence maximum of chlorophyll a (680 nm) and phycocyanin (656 nm), respectively. For SFS averaging was carried out over 10–15 cells.

eight obtained spectra are presented in **Figure 4**. Then, the integral spectra of the whole culture were obtained at corresponding excitation wavelengths using Cary Eclipse fluorimeter (Varian Cary) (**Figure 4d–f**; green lines). Two sets of eight fluorescence spectra for cells in normal and depressed physiological state were taken to obtain a linear combination for fitting procedure a set of integral spectra. Each spectrum in two sets was averaged over 10–15 cells. It should be noted that to raise accuracy of calculations, the curve fitting process was done over the whole set of the eight spectra simultaneously, so that to exclude any ambiguity. The fitting coefficients for alive and depressed cells were about 2 and 1, correspondingly. Thus, the whole culture can be considered as a healthy and being in the exponential or logarithmic growth phase.

As it is following from the plots of **Figure 4a–c**, the degree of cell damage is determined mostly by the relative fluorescence intensity of phycobilins at 656 nm and chlorophyll-binding proteins at 680 nm. It is especially clear from the excitation wavelength 488 nm (**Figure 4b**), where the fluorescence spectra for healthy and depressed cells have the mirror shape. For the 458 nm excitation, the shape and the intensity of fluorescence spectra for this two states differ not so much because this wavelength excites better the peak of chlorophyll-binding proteins in PS II (680 nm), which is not affected. On the other hand, the 514 nm wavelength strongly excites the fluorescence of the antenna pigments and the fluorescence spectra intensity differs significantly for the healthy and depressed cells.

Despite the near qualitative character of the presented analysis, the accuracy and stability of this method are ensured by the simultaneous calculations over a series of eight spectra. If several variants are possible while fitting one spectrum, then simultaneous fitting of eight spectra will provide a sufficient accuracy. This small example demonstrates that the shape of single-cell fluorescence spectra reflect the physiological state of cyanobacteria and the sum of single-cell contributions represents viability of the whole culture because these phenomena are strictly related with the correct or incorrect functioning of the photosynthetic apparatus.

Finally, it should be noted that the presented technique can be modified for obtaining any developmental stage of the selected cyanobacterial culture, by considering the reference set of fluorescence spectra to be fitted. For instance, in the technological process of the industrial incubation of cyanobacteria, involved in food production, it is quite desirable to estimate the optimal stage of the culture development when the accumulation of biologically active compounds attains its maximum. This process can be controlled via online recording of several fluorescence spectra and comparing them with reference ones. Moreover, in the environmental monitoring, the online estimation of the viability of the cyanobacterial colonies in the field samples via fast and effective fluorescence technique can assist the prediction and prevention of the hazardous cyanobacterial blooms.

More detailed description of the presented technique and its application can be found in [83].

3.2. Ultrasonic treatments

In recent years, several environmentally friendly methods for preventing of toxic cyanobacterial "blooms" of water bodies have appeared. One of them is a weak ultrasound treatment. Unfortunately, due to the low intensity of the applied sonication and its constant but weak influence on the biophysical parameters of the cyanobacteria most of the results of the previous investigations are quite ambiguous [32–34, 84–89].

Despite the significance of the problem of cyanobacterial blooms and a variety of applied methods to control them, the sufficient principles of investigation and monitoring the results of various external actions on cyanobacteria are not developed. Currently, the most of all studies are based mainly on traditional visual methods of obtaining results or on the analysis of fixed or dissociated environmental samples. However, standard methods can only record the presence of the bloom in reservoir, but cannot determine at what evolution stage it is situated, or, all the more, to predict the possibility of further cyanobacterial bloom. This owes to the use of rather crude methods of monitoring of the physiological state of the culture during the experiment. All previously elaborated monitoring methods either destroy cells or change significantly their physiological state just before the measurements, so it is impossible to determine the initial physiological state of the treated culture.

For example, in the paper [87], the ultrasonic inhibition of *Microcystis aeruginosa* cell growth and extracellular microcystins release was examined. The authors reported the decrease of antenna complexes like cyanobacterial chlorophyll a and phycocyanins (PC), and the oxygen evolution rate. The conclusion about slowing down of the photoactivity and damaging of the antenna complexes was made according to the measuring of the growth rate of the whole culture. This is not the case because there is no any confirmation that the single-cell physiological state really

changes. May be the whole culture died due to other reasons or simply several cells were destroyed during sonication. Moreover, the pointed sonication power settings (about 0.32 W/mL) cannot be considered as a weak and environmentally friendly treatment. It cannot be applied to the natural reservoirs, so that to carry out a real control on cyanobacterial blooms in open water. Obviously, this high intensity of sonication was used to obtain any possible results because used methods are not precise enough. Thus, this example shows that the standard methods do not give the correct results, and new precise, nondestructive *in vivo* methods for monitoring of the physiological state of cyanobacterial cultures are required for such investigations.

In the previous section, we present a novel noninvasive technique for estimation of the physiological state of single living cyanobacterial cells. Let us demonstrate the results of the application of this technique to the ultrasonic treatment experiments with cyanobacterial cultures.

In this investigation, the photosynthetic activity of living cyanobacterial cells treated by ultrasonic radiation was studied. A strain *Synechocystis CALU 1336 aquatilis* from CALU collection of core facility Center for Culture Collection of Microorganisms of St. Petersburg State University was used in the experiment. After 8 days incubation, the tested culture was divided into two parts and placed in the same light, temperature and nutrient conditions. One part was a control sample and another part was sonicated via original, specially designed, ultrasonic laboratory device shown in **Figure 5**.

The sonication was performed in a 35-mm plastic Petri dish placed on the ultrasound emitter. Ultrasound emitter consists of a ceramic resonator connected with handheld pocket frequency generator HPG1 (Velleman Instruments Inc.), it has an emitting surface area about 13 cm^2. The ultrasonic field inside the dish was measured out with a standard calibration ultrasound-needle-hydrophone connected to a TDS 3000 oscillograph (Velleman Instruments Inc.). For each experiment, 10 mL cyanobacteria solution was filled in a dish and kept at 25 ± 2°C. The ultrasound frequency and power density were about 60 kHz and 5.85 mW/mL, correspondingly, and the sonication time was near 24–30 h. Such sonication conditions were

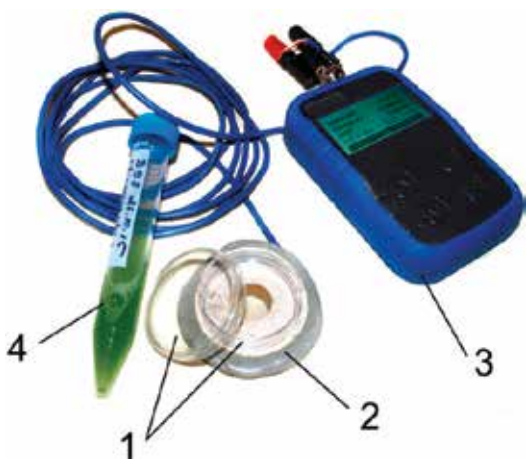

Figure 5. The developed device for laboratory ultrasonic treatment of cyanobacterial culture. (1) Plastic petri dish, (2) ultrasonic emitter, (3) generator of ultrasonic frequencies and (4) sample culture.

chosen following the literature reports [32, 86] and based on our preliminary tests. During sonication, the cyanobacteria solution was carefully shuffled and taken for analysis just in the process of sonication. Control sample without any treatment was kept under exactly the same conditions during the whole period of the experiment.

In **Figure 6**, single-cell fluorescence spectra for two emission wavelengths (488 and 543 nm), obtained by standard lambda-scanning using Leica TCS-SP5 CLSM, are shown for control and treated samples. Blue lines demonstrate the spectra of the control suspension, whereas red lines show the spectra of the experimental one. It is clear that the fluorescence intensity in the control group differ from the experimental one. According to the preliminary studies, enhancement of the fluorescence at 660 nm in the experimental culture indicates that this culture is in a depressed physiological state in comparison with the control group. Each spectrum in **Figure 6** was obtained by averaging over 15 cells. Spectral analysis on the cell level using CLSM makes it possible to obtain more comprehensive information on the small variations in physiological state of both single cells and the entire culture exposed to ultrasonic treatment. During ultrasonic treatment, single cyanobacterial cells were studied at the first day to obtain the reference spectra of the initial cells in a good physiological state. Then, after 24–30 h, both control and treated specimens were studied and their fluorescence spectra were compared (**Figure 6**).

Let us mention here again that the treated and control probe initially were taken from one cultural sample and were placed at the same environmental conditions. Else, all the obtained results were double controlled via conventional fluorometric methods such as pulse amplitude modulation (PAM) and absorption and fluorescent characteristics of the whole culture [90]. These measurements were conducted using the light curves method on the PAM 2500 pulse spectrofluorimeter (WALZ, Germany) and a standard spectroscopic technique using a Cary Eclipse fluorimeter (Varian Cary), correspondingly. The dependences of the electron transport rate (ETR) and the quantum yield of photosystem PS II (Y(II)) on photosynthetically active radiation (PAR) under blue actinic light, obtained via WALZ PAM, show that ETR and

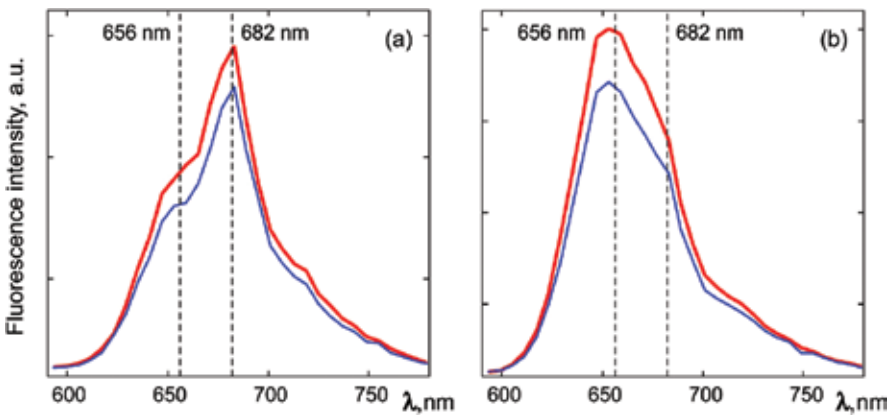

Figure 6. Single-sell fluorescence spectra for cyanobacterial strain Synechocystis CALU 1336 obtained at two excitation wavelengths (a) 488 nm, (b) 543 nm. Red lines—the culture exposed to ultrasound; blue lines—the control culture. Each spectrum was obtained by averaging over 15 cells.

Y(II) decrease in the sonicated culture, which indicates that the physiological state of the culture under sonication is depressed. At the same time, the nonphotochemical quenching of the absorbed light by the fluorescence rises considerably for the treated culture. Comparison of the results of the CLSM spectroscopic measurements with those obtained using conventional fluorimeter and pulse-amplitude modulation approaches confirmed the inhibitory effect of low ultrasonic frequencies (~60 kHz) on the physiological state of cyanobacterial cells and whole cyanobacterial cultures.

It can be concluded with confidence that the results obtained via the CLSM technique are correct, and the reduction of the photosynthetic activity and dumping of single-cell physiological state occur as a reply on the external ultrasonic action. The results presented here demonstrate the experiments conducted with the strain Synechocystis CALU 1336 aquatilis; however, similar results were already obtained for another unicellular cyanobacterial species (*Microcystis CALU 398*). Thus, the treatment presented here may refer to a rather diverse group of unicellular cyanobacteria.

It should be noted that ultrasonic treatment is widely used not only for inhibition of cyanobacteria growth diring harmful blooms, but also for enhancing of protein content and the whole biomass in the industrially cultured strains [91, 92], depending on power-frequency characteristics. Thus, it is very important to obtain on-line correct information about the influence of the ultrasound with given power and frequency on the specified cyanobacterial strain. The noninvasive fluorescent technique presented here gives the opportunity to detect any weak variations in the physiological state of single cyanobacterial cells in real time during sonication.

3.3. Differentiation of cyanobacterial cultures on the base of single-cell fluorescence spectra

The automatic on-line differentiation of cyanobacterial species is a key problem in both industrial biomass production and environmental monitoring. In this section, we present a novel technique for taxonomic discrimination of cyanobacteria based on the numerical analysis of *in-vivo* single-cell fluorescence spectra. An optimal set of the parameters is considered, which is sufficient for determination of the taxonomic position of cyanobacteria by means of mathematical statistics. On the base of the linear discriminant analysis obtained spectroscopic data for 21 cyanobacterial strains from *CALU* collection were analyzed. It was shown that the presented technique allows an accurate differentiation of cyanobacteria up to the species/strain level and enables to distinguish automatically potentially harmful strains.

Since the early 1950s, three different methods are commonly used to characterize phytoplankton and cyanobacterial samples taxonomically: high performance liquid chromatography (HPLC) [13–15, 23, 93]; flow cytometry [10, 11, 94, 95]; and optical microscopy. Various methods have been developed with the aims of increasing accuracy and yielding qualitative information. However, all of them have different limitations. Till now, the best taxonomic differentiation is still obtained using classical inverted microscopy. Unfortunately, this method is time-consuming, human-based and requires appropriate technical skills, and this eliminates the possibility of its application for continuous on-line monitoring. Nearly single-cell flow cytometric analysis is based on light scattering by the cells and fluorescence of the chlorophylls and the phycobilins. It can be easily automated, but it is appropriate only

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for unicellular species and is useless for numerous industrially cultured filamentous strains. HPLC is the only method, of the three, that is based on the chemical constituents in the sample. The problem is that during the chemical sample preparation, most of the information about the peculiarities of individual species is lost and the residual part of the information is not enough for species/strain classification inside cyanobacterial genera, and is suitable only for the rude differentiation of big classes of phytoplankton. As it was mentioned earlier, several factors contribute to the spectroscopic properties of the phycobilins: the number and chemical nature of the bilins attached to the polypeptide chains; the effects of protein conformation or aggregation state; and interaction between the bilins. Any of this feature can be unpredictably changed during the extraction and purification procedure [96]. Thus, only spectroscopic properties of the intact living cells can give pure unspoiled information about distinctive features of light harvesting complex in specified cyanobacterial strain.

Analysis of the *in-vivo* absorption and fluorescence spectra is an alternative way of obtaining qualitative information about the phytoplankton abundance and composition, which is continuously demonstrated by various publications [21, 23–27, 36, 97–100]. The relative phytoplankton abundance can be calculated once initial assumptions about the phytoplankton classes present and their pigment compositions have been made [10, 24, 25, 36, 100]. However, the correct classification of cyanobacterial species on the base of their fluorescence signature was hampered by alterations in pigment composition within one strain, which depends on the environmental conditions [93]. On the other hand, several researchers show that the nutrient and light limitations do not significantly change the initial fluorescence spectra and cannot impede the species discrimination [98, 101].

May be the first attempt to use phycoerythrins as chemotaxonomic markers was done by Glazer et al. [96] for red algae in 1982, but until now fluorescence spectra of phycobilins do not appear to be useful at familial, ordinal and class levels in taxonomic studies. Although the investigation in [96] concerns only purified high molecular weight phycoerythrin from red algae this work clearly demonstrates the possibility of the correct taxonomic analysis on the base of phycobiliproteins structural differences, which can serve as intrinsical fingerprints for taxons and genera in phytoplankton diversity. Later the correlation between the distribution of the biliproteins and the genera of *Cryptophyceae* was discussed in [102]. In 1985, Yentsch and Phinney [19] proposed an ataxonomic technique that utilized the spectral fluorescence signatures of major ocean phytoplankton. Seppälä and Olli [97] used spectral fluorescence signals to detect changes in the phytoplankton community. In 2002, Beutler et al. reported a reduced model of the fluorescence from the cyanobacterial photosynthetic apparatus designed for the in-situ detection of cyanobacteria and presented a commercially available diveable instrument for on-line monitoring of phytoplankton structure [21].

We elaborate a strict procedure for recording and processing single-cell fluorescence emission spectra, which eliminates the most of mentioned above difficulties and has a quite high classification accuracy. As well as according to our technique the fluorescence spectroscopic information is obtained via CLSM, the initial data has less variations and can be accurately sorted. Any objectionable and unpredictable impact can be eliminated at the first step of obtaining fluorescence spectra. Since noninvasive and nondestructive method is used the information about vital cell operation (e.g., light harvesting) can be additionally taken into account. All this allows one to obtain the desirable result directly following the procedure.

The classification procedure consists of three steps: (1) obtaining single-cell fluorescence spectra and creation of reference database; (2) data processing and extraction of classification parameters; (3) statistical analysis and evaluation of classification procedures.

Step 1. To illustrate the usual form of spectra in Ref. database, in **Figure 7** several characteristic sets of single-cell fluorescence spectra are presented. Here four sets related to four cyanobacterial strains are shown: *Microcystis CALU 398*, *Merismopedia CALU 666*, *Leptolyngbya CALU 1715* and *Phormidium CALU 624*. Cyanobacterial strains are labeled according to *CALU* collection of the Core Facility Center for Culture Collection of Microorganisms of Saint-Petersburg State University. Each spectrum in the set was obtained by means of CLSM Leica TCS-SP5, using corresponding laser-line for excitation (405, 458, 476, 488, 496, 514, 543, and 633 nm). Corresponding excitation wavelengths are given over each spectrum. All spectra are normalized to the maximum intensity and shifted along x-axis for the clarity of observation. Four characteristic wavelengths, corresponding to the fluorescence maximum of different pigments can be easily distinguished at each spectrum: (1) peak near 580 nm corresponds to the fluorescence of phycoerythrin (is absent for *Microcystis* and *Leptolyngbya*), (2) peak near 656–560 nm corresponds to the fluorescence of phycocyanin and allophycocyanin in common (they are undistinguishable at room temperature), (3) peak near 682 nm corresponds to the fluorescence of chlorophyll *a* of PSII, and (4) peak or shoulder near 715 nm represents the fluorescence from PSI.

To obtain the representative fluorescent signature for given cyanobacterial strain by means of CLSM several points should be kept in mind. Each set of fluorescent spectra for single cell includes 4–8 spectra, obtained at different excitation wavelengths. One or two spectra in series is not enough for further differentiation. Thus, the low power settings should be used at all laser lines in order to eliminate cell damage during the record. Moreover, the excitation

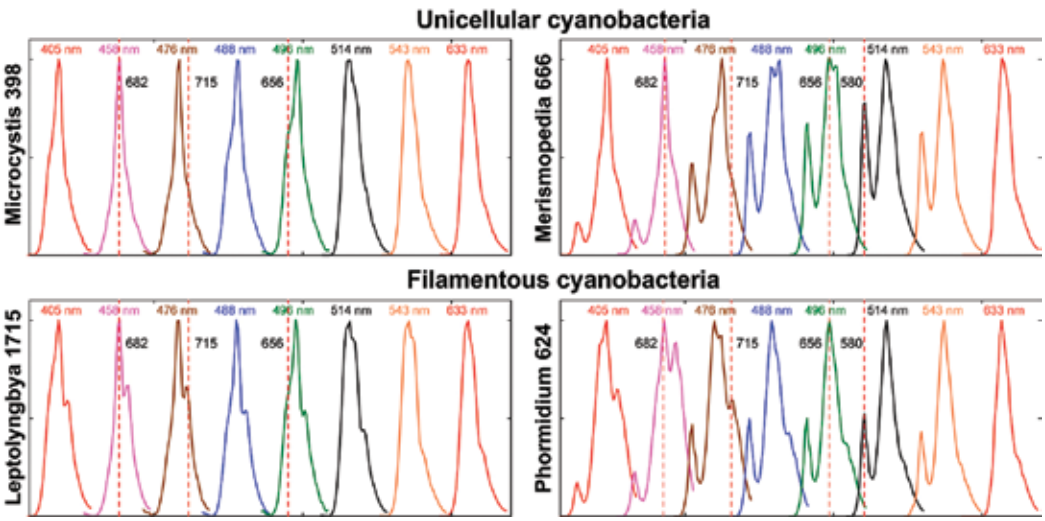

Figure 7. Four characteristic sets of single-cell fluorescence spectra, corresponding to unicellular and filamentous cyanobacterial strains. The excitation wavelengths (405, 458, 476, 488, 496, 514, 543, and 633 nm) are given over the curves. All spectra are normalized to the maximum intensity and shifted along x-axis for convenience of observation.

wavelengths, that excite mostly one pigment (514, 543, and 633 nm), give less information than other laser lines and can cause more damage due to over-excitation. So, such excitation wavelengths should be used at the end of the record. To create a reference database of fluorescence spectra only the cells in normal physiological state should be used (if the study of the depressed physiological state is not a case of current investigation).

If the database is formed from the cultured species, it is desirable to obtain reference spectra several times at different days and for various developmental stages of the culture to exclude any discrepancy and to take in account all possible variations in spectrum shape. The experimental sampling for each strain should include the sets of fluorescence spectra for more than 30–50 cells, to evaluate the statistical analysis. For the cultured species or for the strains from culture collection specified nutrient, temperature and light conditions should be applied, identical for all samples involved in classification. This is strongly required to exclude any adaptation effects.

If the investigation is conducted over natural samples (for environmental monitoring), the reference database should be recorded for each tested reservoir because the difference in nutrient and light conditions could change the initial fluorescence spectra considerably. Moreover, this database should be extended by new experimental data constantly. While the reference spectra are available, the routine environmental monitoring is acceptable.

The whole procedure of obtaining intrinsic single-cell fluorescence spectra used in this study was designed to minimize preparatory manipulation, so as to conduct a noninvasive investigation of small amounts of experimental material and to prevent any damage of living cells.

Step 2. While as the initial database is completed, the extraction and selection of classification parameters is carried out. To extract from the initial single-call fluorescence spectra, a set of classification parameters recently a computer program in the MATLAB software application [103] was elaborated. By means of this program, interpolation and smoothing of the raw spectra were carried out to eliminate the random noise and metering fluctuations. All spectra were reduced to the same scale and size of data array by the procedure of normalization and extrapolation. The first derivative was taken over smoothed and interpolated spectra and the fast Fourier transform was performed to exclude random noise, owing to the low intensity of the exciting and emitting energy. Else, some specific values characterizing the shape of the curves and the spectral composition of their derivatives were calculated, such as asymmetry and excess.

In **Figure 8**, several plots are given to illustrate the process of extracting parameters. At the left panel, normalized characteristic *in-vivo* single-cell fluorescence emission spectra at excitation wavelength 488 nm for two cyanobacterial species *Leptolyngbia CALU 1713* (blue lines) and *Nostoc CALU 1817* (red lines) are presented. Shaded regions mark the ranges of averaging for corresponding fluorescence intensities (575–586; 654–658; 679–685; and 714–723 nm). While getting the mean values at the corresponding area, one can calculate the impact of each region into the whole fluorescence intensity in percents. This will be the first set of classification parameters named fluorescence emission percentage contribution for individual pigments. For eight laser lines and for four zones as a result, we have a set of 32 parameters. The usual parameters for characterizing the shape of the curves asymmetry and excess (AE)—give another 16 parameters. Finally, the last set—the FFT-contributions in three specified regions of wavevector domain—is formed analyzing corresponding Fourier-transforms for each first derivation

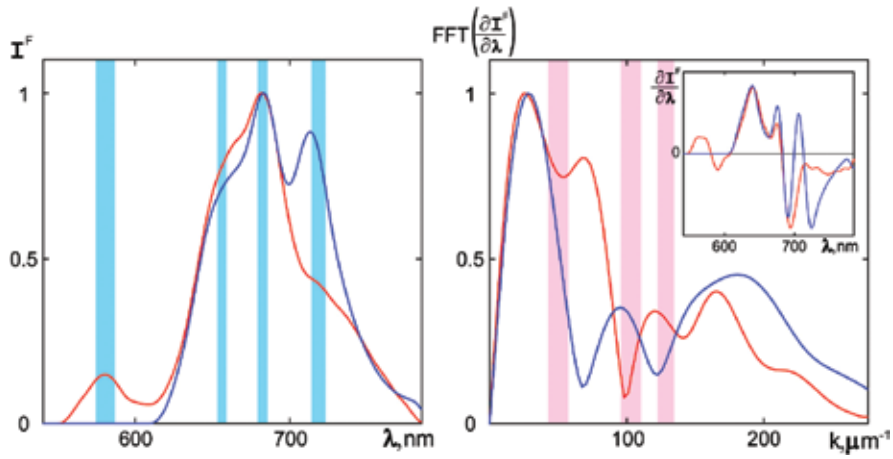

Figure 8. The illustration for the classification parameter's calculation. Left panel: normalized in-vivo single-cell fluorescence emission spectra of two representative cyanobacterial species: blue lines—*Leptolyngbia CALU 1713*, red lines—*Nostoc CALU 1817*. Right panel: Fourier transformants normalized at maximum for the corresponding first derivations of the initial fluorescence spectra. In the inset the first derivation curves are plotted. Excitation wavelength 488 nm. Shaded regions show the bands of averaging.

plot (see **Figure 8**, left panel). The inset in **Figure 8** shows the first derivation curves for corresponding spectra. Three regions (43–58 μm^{-1}; 95–110 μm^{-1}; and 123–135 μm^{-1}) were chosen and the mean value inside each was calculated. Thus, the last set of the parameters includes 24 values for each observation. Finally, for each spectra set, we extract 72 parameters, which are quite enough for evaluating classification by means of the linear discriminant analysis.

It should be noted that this procedure of the parameter's extraction varies according to the obtained data. The set of extracted parameters presented here is only an example of successful solution of formulated classification problem. Moreover, after statistical data analysis by means of hierarchical cluster analysis and stepwise linear discriminant analysis, the extracted set was reduced to 57 items to exclude a strict correlations between the parameters.

Step 3. The last step includes the evaluation of classification procedure. Here we apply linear discriminant analysis (LDA), which is well known, and often applied to various biological objects. The procedure involved creating linear combinations of parameters with normal errors that best discriminate between site groups of cyanobacteria defined *a priori*. LDA was performed with the computer program designed in the MATLAB software [103], in which combinations of initial parameters were selected to maximize the ratio of group means discriminant scores to within-group variance [99, 104].

For this investigation, 314 sets of 8 spectra corresponding to 21 strains and 15 genera of cyanobacteria from the *CALU* collection were used. The results of Fisher discriminant analysis evaluated over 57 parameters is presented in **Figure 9**. The upper panel show 3D-plots in the space of discriminating functions. It is clear that the discrimination between species is sufficiently good. Moreover, the closely related species (e.g., *Spirulina* and *Oscillatoria*, *Synechococcus* and *Chlorogloea*, *Microcystis* and *Myxosarcina*) appear close to each other. Such species as *Leptolyngbia*, *Geitleninema* and *Oscillatoria*, which includes several strains, form a big groups. However, inside these groups, single strains also can be discriminated. In the lower

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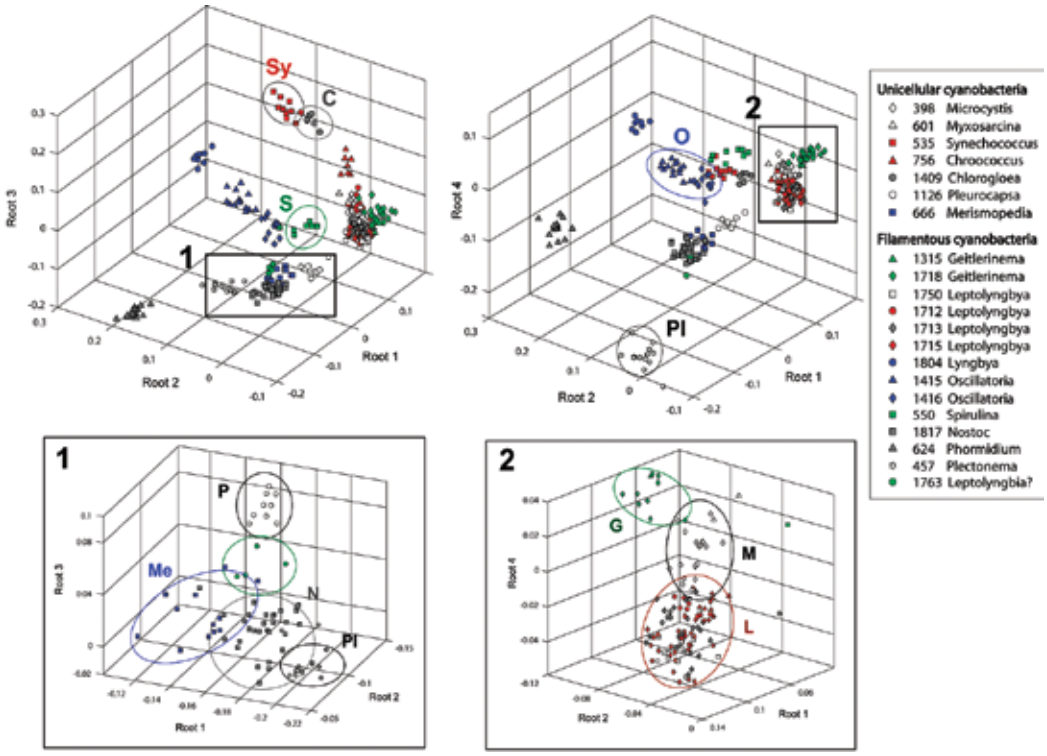

Figure 9. The results of linear discriminant analysis. Solid curves bounded the regions, occupied by specified species: G—*Geitlerinema*, L—*Leptolyngbya*, O—*Oscillatoria*, M—*Microcystis*, Me—*Merismopedia*, P—*Pleurocapsa*, Pl—*Plectonema*, N—*Nostoc*, S—*Spirulina*, C—*Chlorogloea*, Sy—*Synechococcus*. The lower panel shows the scaled view of the corresponding regions.

panel, the corresponding scaled regions are presented. In the legend, all used cyanobacterial strains are named and enumerated according to *CALU* collection. Solid curves bounded the regions, occupied by specified species: G—*Geitlerinema*, L—*Leptolyngbya*, O—*Oscillatoria*, M—*Microcystis*, Me—*Merismopedia*, P—*Pleurocapsa*, Pl—*Plectonema*, N—*Nostoc*, S—*Spirulina*, C—*Chlorogloea*, Sy—*Synechococcus*.

The classification accuracy in the presented example is near 98.3%. However, for the limited set of laser lines (4 instead of 7) and classification parameters (24 instead of 57), the classification accuracy does not reduce considerably—93.7%. The high classification accuracy is due to the fact that LDA works with distribution functions for classification parameters and their statistical characteristics, which allows to build a good classification model.

It should be noted here that the limited set of laser lines and classification parameters was considered subjecting to further possible application of the presented technique in the microelectronic device for on-line data collection in environmental monitoring. During this investigation, the possibility of the implementation of data collection and data processing in one software-hardware application device on the base of on-a-chip technology was examined. Obviously, for the production of such device, it is quite desirable to have less radiation emission sources and limited number of detection spectral ranges.

The universality of the considered technique makes it possible to use it for investigation of any phytoplankton species irrespective of their habitat or cultivation. Utilizing data from several fluorescence spectra, instead of one, results in more fingerprint information which leads to the taxonomic differentiation on a finer scale. Classification procedure, presented here, was carried out by means of statistical analysis on the base of mathematical characteristics of intrinsic fluorescence spectra of living single cells; therefore, it is free from usual subjectivity, which can occur while using methods of direct optical microscopy. Moreover, formalization of data processing gives a wide opportunity for automating of the classification procedure of cyanobacterial strains in field samples, while on-line monitoring of water bodies is conducted.

Undoubtedly, the data set should be expanded to include more species and phytoplankton classes/divisions, grown under different nutrient and light conditions. However, this work already demonstrates the potential of the discrimination of phytoplankton classes by fluorescence microscopic spectroscopy. This work lays the foundation for determining cyanobacterial abundance by direct fluorescence measurement of sea- and freshwater. Combining the knowledge of phytoplankton structure along with taxon-specific measurements of photosynthetic activity and biochemical cell composition, can lead to new models which increase the reliability of on-line monitoring.

4. Conclusion and outlook

In this chapter, the most powerful CLSM method for investigation of cyanobacteria—the fluorescence microscopic spectroscopy—was presented. This method allows to study living cyanobacterial cells via noninvasive and nondestructive technique and obtained in-vivo information about weak variations in single-cell functioning. It should be noted that CLSM provides some other interesting techniques, which can give deep insight into physiological processes that rules cyanobacterial life, such as FRAP [68–69, 105, 106] and hyperspectral microscopy [44]. FRAP is used to measure the mobility of phycobilisomes in intact cyanobacterial cells and hyperspectral microscopy helps to determine pigment localization and distribution in living cyanobacterial cells. Moreover, several time-dependent techniques for investigation of the dynamic properties of photosynthetic apparatus of cyanobacteria, such as PAM, can be implemented at a single-cell level by means of CLSM.

A limited number of examples, presented here, of possible fluorescence microscopic spectroscopy implementation (e.g., the investigation of biological diversity and monitoring of physiological state of cyanobacterial cultures) can significantly rise an effectiveness of the routine procedures in environmental monitoring and industrial culture production. Confocal microscopic spectroscopy gives a unique opportunity to introduce automation into these processes.

On the other hand, the indirect application of the presented results of the single-cell spectroscopic investigations can give a new information to improve remote sensing control. Spectral information recorded by satellite-carried sensors is already used for mapping of algae distribution, and due to the high frequency of data collection provides a database for estimation of phytoplankton dynamics over large areas [107]. Presented investigation gives an opportunity to control also cyanobacterial communities. The elaborated technique can be supported with algorithm that includes a new mathematical fitting strategy which automatically can

cope with the environmentally caused variations of the cyanobacterial fluorescence spectra. Moreover, an additional fluorescence information on the physiological state of cyanobacterial cultures provides a new information for predictive modeling and aquatic management, alternatively to the delayed fluorescence described in [108].

The formalization of the genera identification and cultural physiological state analysis give an opportunity to develop a compact on-a-chip nanoelectronic device for preliminary on-line investigation of the field samples in situ and in vivo and for controlling of the laboratory cultures during industrial incubation.

Obviously, the proposed methods require further development, including evaluation of more species representing more phytoplankton classes, and including non-taxonomic features, such as photoadaptation. Although the quantitative measurements were not performed in this study, they could be possible while all stages will be standardized. However, this work already demonstrates a high potential of fluorescence microscopic spectroscopy. We suggest that CLSM methods have potential application for several of the approaches noted earlier and also other studies regarding photosynthetic apparatus of cyanobacteria. We hope that the unique cell-biology of cyanobacteria will encourage further investigations because of their growing importance in rural biotechnology and commercial production.

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Chapter 3

Diversity of Cyanobacteria on Limestone Caves

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Abstract

The caves are the biodiversity centers for different types of microorganisms, especially for cyanobacteria. They are also present in almost all extreme environments, and their importance in terrestrial ecosystems is greater because of the decreased competition from vascular plants. Cyanobacteria occurring on rocks are epilithic (colonizing the substrate surface), hypolithic (growing under pebbles and small stones), and endolithic (present in an upper layer of rock). There are three limiting factors for cyanobacteria growing in caves: light or its lack, high humidity, and constant temperature. In caves, one can find not only the cosmopolitan cavernicolous species but also rare taxa. Light, transmission, and scanning electron microscopy (SEM), laboratory cultures, as well as molecular phylogenetic studies are important tools in the study of cave cyanobacteria.

Keywords: cyanobacteria, cave, diversity, ecology

1. Introduction

Caves are highly specific environments scattered all over the world, and karst caves are considered a specific case of extreme environment [1, 2]. Caves are also biocenters for biological studies, and they are characterized by low natural light, uniform temperatures throughout the year, and high humidity [3]. A typical cave is described as having three major habitat zones based on light penetration and intensity: the entrance, transition, and dim light zone. Lamprinou et al. [2, 4] and Roldán and Hernández-Marinė [5] suggested that caves are the most typical and well known of the different karstic formations geologically created in limestone substrates and even caves with dim natural light were found to host phototrophic microorganisms. Lee et al. [6] and Falasco et al. [7] stated that biology of caves is not only a

matter of exploring unique and extreme ecosystems but also understanding of the ecological balances on Earth. Unlike other nutrient-deprived environments where one might study oligotrophic conditions, most caves are readily accessible. The absence of photosynthetic organisms leads cave cavernicolous species to depend on allochthonous resources or, in rare cases, on chemoautotrophic bacteria and roots [8]. Allochthonous resources are carried by water, wind, gravity, or animals that are translocating between caves and external environments; thus cave communities are highly dependent on the adjacent epigean environments [3, 9]. Cave ecosystems are characterized by stable conditions, although cave habitats are extremely oligotrophic, receiving limited supplies of organic matter. Poulson and Lavoie [10] determined that organic matter in caves derives from plants and guano, whose bioavailability is largely dependent on its chemical properties and on environmental physical factors, such as temperature and light. Falasco et al. [7] described cave biological microorganisms as characterized by presence of resident and nonresident organisms (accidentals), which enter caves occasionally via water, sediments, wind, or air, as spores, or even carried in by animals. On the other hand, caves generally can be described as having relatively low species richness, biomass, and density, and where cavernicolous species are dominated by cyanobacteria, which are the first colonizers [11]. Such cave conditions attract cosmopolitan species, leading to the gradual elimination of particularly sensitive native species [12]. However, most caves, at least in Europe, are damp and the walls at the entrance are covered with a blue-green cyanobacterial gelatinous mass [11]. Entering a cave, one goes through a series of zones: entrance (strongly influenced by surface conditions), deeper, and the dim light zone. Deep in a cave, there is an absence of light, temperature at or near the MAST (mean annual surface temperature) for the region, and high humidity. At the entrance of limestone caves and on surfaces around an artificial lighting (caves made accessible to general public), cyanobacteria compete for light with algae, mosses, and sometimes also prothalli of ferns and flowering plants, but in the deepest recesses of caves, where the light is reduced to a minimum, there are only phototrophs [13, 14]. Hoffmann [15] divided species occurring in caves into three categories based on their ecological evolutionary: troglobiotic (obligatory cavernicolous that cannot survive outside the cave), troglophilic (growing and reproducing in caves), and trogloxenic (accidentally appearing in the cave environment). Lamprinou et al. [4] suggested that very few cyanobacteria are considered as obligatory cavernicolous such as *Geitleria calcarea, G. floridana, Herpyzonema pulverulentum*, and *Symphyonema cavernicolum.* However, Hoch and Ferreira [16] and Rabelo et al. [9] stated that the cave environments favor scenarios of high endemism, where species can be restricted even to a single cave. This high endemism leads several species to be threatened, particularly in tropical regions where the demand for mineral resources, food production, and energy generation has grown due to rapid development and population growth [17].

Cyanobacteria, the oldest cellular organisms on Earth, are very resistant to severe conditions in cave, including even darkness. Moreover, the cyanobacteria are able to survive in dark caves, as heterotrophic organisms, and are consumed by cave palpigrades [18]. It is interesting that cyanobacterial species, as suggested by Barton and Jurado [19], adapt to cave environment by interacting with minerals there and some of processes reshaping the mineral structure of the cave walls, floors, and ceilings—for instance, by forming speleothems such

as stalactites and stalagmites. One of the most interesting problems, although not precisely known, is the origin of the global (cosmopolitan) distribution of cavernicolous cyanobacteria.

1.1. Study area

Europe is an exceptional continent for its abundance and variety of subterranean karstic forms comprising the natural geological heritage, and therefore many caves are protected and European countries have programs for the protection of caves and geodiversity. Some caves can be visited and are either ecologically or culturally a tourist attraction, and are important centers of mass touristic exploitation. While numerous caves can be visited all year round, others are difficult to get to and are explored only by speleologists and wild animals.

2. Material and methods

Cyanobacteria samples were collected from walls and ceiling of limestone caves where evidence of biological colonization was present. Algal crusts were scraped from the stone substrata (**Figure 1**) using nondestructive adhesive tape method also by removing the biofilm with sterilized scalpel, stored in labeled sterile plastic bags and used directly for observation under a light microscope (LM) or as inoculate for cultures on agar plates. Cyanobacterial material was cultured on agar plates or liquid medium in lab, and in consequence, filamentous and coccoid species were obtained. The samples were studied using a light microscope at 100× objective with oil immersion. Photomicrograph was taken using photomicroscope equipped with a digital camera.

For transmission electron microscopy (TEM), samples fixed in glutaraldehyde were postfixed in an osmium tetroxide solution, dehydrated in a graded ethanol series, and embedded in epoxy resin. Thin sections were collected on copper grids and stained with uranyl acetate and lead citrate. For scanning electron microscopy (SEM), cleaned sample was dried onto a coverslip, attached to an aluminum stub, and then gold coated, whereas environmental variables (temperature, light, and humidity) were measured using digital thermohygrometer and photoactinometer. For each parameter, the mean value with standard error was calculated.

Figure 1. Dark-green—colored cyanobacteria patches on walls in Sąspowska cave (Ojców National Park, Poland).

3. Results and discussion

Cavernicolous cyanobacteria belong to the orders Chroococcales, Nostocales, Oscillatoriales, and Stigonematales. Chroococcales are the most common order represented by genera: *Aphanocapsa, Aphanothece, Chondrocystis, Chroococcidiopsis, Chroococcus, Gloeocapsa, Gloeocapsopsis,* and *Gloeothece.* Whereas among Nostocales were present only three genera: *Hassalina, Nostoc,* and *Scytonema.* Coccoid and filamentous cyanobacteria species were dominant in the entrance zone, while in the dim light zone they were encountered only sporadically. Czerwik-Marcinkowska and Mrozińska [20] and Martínez and Asencio [21] stated that coccoid forms are more abundant in dark areas, whereas filamentous forms tend to be more diverse in illuminated part of cave, unlike the findings of Vinogradova et al. [22]. Epilithic cyanobacteria are the first colonizers and play an important role in the genesis of biofilms, being able to produce exopolymeric substances (EPS) that allow the adhesion to rocks and the consequent establishment of a microbial community [7]. Besides the colonization of various stone substrata and the production of pigments that are responsible for colored effects on rocky cave walls and erosion of the stone substrata, they can also serve as a food source for animals. Almost all cavernicolous cyanobacteria have gelatinous extracellular sheath layers of various thickness composed of polysaccharides. Keshari and Adhikary [23] observed that the gelatinous extracellular sheath of cyanobacteria plays a crucial role in adhesion to the substratum and also acts as a water reservoir, thus enabling the cyanobacteria to survive drought periods. Pattanaik et al. [24] suggested that water stress proteins, glycan, and UVA/B absorbing pigments are the main components of the EPS of cyanobacteria. The genus *Gloeocapsa* has the most various colorations due to the presence of a pigment called gloeocapsin. Another well-studied pigment, scytonemin, causes the dark coloration of cyanobacterial crusts [24]. Genera that usually dominate dark-colored crusts are *Scytonema, Nostoc,* and *Tolypothrix*. Some cyanobacteria from genus *Scytonema* have calcified trichomes [25]. In **Table 1**, the most frequent and abundant cyanobacteria species found in different European caves based on literature results since 2010 are reported.

3.1. Ecology of cyanobacteria in caves

Whitton [25] stated that cyanobacteria have existed for 3.5 billion years and they are the most important photosynthetic organisms on the planet for cycling carbon and nitrogen. Cyanobacteria living in limestone caves present a unique group of microorganisms, which developed adaptations against the more or less extreme conditions of their habitats. They play an important role in several aspects of the environment such as colonizers, nitrogen fixers, prey for micrograzers, or deterioration agents. Cyanobacteria are morphologically diverse group of prokaryotes successfully colonizing and inhabiting almost all kind of terrestrial and aquatic habitats including extreme microhabitats such as caves, rocks, external walls of monuments, and buildings [26, 27]. Wild caves and caves made accessible to general public are characterized by extreme conditions, and they also offer a unique habitat for cyanobacteria [20]. Cyanobacteria are prone to environmental stress such as desiccation, temperature, and UV radiation, and they adopted survival strategies by producing

Cyanobacteria	Origin	References
Aphanocapsa fusco lutea	Božana Cave (Serbia)	Popović et al. [28]
Aphanocapsa muscicola	Božana Cave (Serbia)	Popovć et al. [28]
Aphanothece saxicola	Gelda Cave (Spain), Božana Cave (Serbia)	Martínez & Asencio [21], Popović et al. [28]
Asterocapsa divina	Gelda Cave (Spain)	Martínez & Asencio [21]
Calothrix fusca	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Chondrocystis dermochroa	Božana Cave (Serbia)	Popović et al. [28]
Chroococcidiopsis kashayi	Božana Cave (Serbia)	Popović et al. [28]
Chroococcus ercegovicii	Božana Cave (Serbia), caves from Ojców National Park (Poland)	Popović et al. [28], Czerwik-Marcinkowska et al. [26]
Chroococcus pallidus	Božana Cave (Serbia)	Popović et al. [28]
Chroococcus spelaeus	Gelda Cave (Spain)	Martínez & Asencio [21]
Chroococcus turgidus	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Chroococcus westii	Gelda Cave (Spain)	Martínez & Asencio [21]
Cyanobacterium cedrorum	Gelda Cave (Spain)	Martínez & Asencio [21]
Cyanosaccus aegeus	Gelda Cave (Spain)	Martínez & Asencio [21]
Cyanosaccus atticus	Gelda Cave (Spain)	Martínez & Asencio [21]
Cyanostylon microcystoides	Gelda Cave (Spain)	Martínez & Asencio [21]
Gloeocapsa atrata	Božana Cave (Serbia)	Popović et al. [28]
Gloeocapsa biformis	Gelda Cave (Spain), Božana Cave (Serbia), caves from Ojców National Park (Poland)	Martínez & Asencio [21], Popović et al. [28], Czerwik-Marcinkowska et al. [26]
Gloeocapsa compacta	Božana Cave (Serbia)	Popović et al. [28]
Gloeocapsa lignicola	Božana Cave (Serbia)	Popović et al. [28]
Gloeocapsa nigrescens	Gelda Cave (Spain)	Martínez & Asencio [21]
Gloeocapsa novacekii	Gelda Cave (Spain)	Martínez & Asencio [21]
Gloeocapsa punctata	Božana Cave (Serbia), caves from Ojców National Park (Poland)	Popović et al. [28], Czerwik-Marcinkowska et al. [26]
Gloeocapsa rupicola	Gelda Cave (Spain), Božnana Cave (Serbia), caves from Ojców National Park (Poland)	Martínez & Asencio (2010), Popović et al. [28], Czerwik-Marcinkowska et al. [26]
Gloeothece cyanochroa	Božana Cave (Serbia)	Popović et al. [28]
Gloeothece palea	Božana Cave (Serbia), caves from Ojców National Park (Poland)	Popović et al. [28], Czerwik-Marcinkowska et al. [26]
Gloeocapsopsis dvorakii	Božana Cave (Serbia)	Popović et al. [28]

Cyanobacteria	Origin	References
Hassalia byssoidea	Božana Cave (Serbia)	Popović et al. [28]
Leptolyngbya carnea	Gelda Cave (Spain)	Martínez & Asencio [21]
Leptolyngbya gracillima	Kastria Cave (Greece)	Lamprinou et al. [2]
Leptolyngbya leptotrichiformis	Gelda Cave (Spain)	Martínez & Asencio [21]
Lyngbya palikiana	Kastria Cave (Greece)	Lamprinou et al. [2]
Nodularia harveyana	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Nodularia sanguinea	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Nostoc commune	Selinits Cave (Greece), Božana Cave (Serbia), caves from Ojców National Park (Poland)	Lamprinou et al. [2], Popović et al. [28], Czerwik-Marcinkowska et al. [26]
Nostoc punctiforme	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Phormidium breve	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Phormidium formosum	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Phormidium vulgare	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]
Pleurocapsa minor	Gelda Cave (Spain)	Martínez & Asencio [21]
Pseudocapsa dubia	Gelda Cave (Spain), caves from Ojców National Park (Poland)	Martínez & Asencio [21], Czerwik-Marcinkowska et al. [26]
Pseudophormidium spelaeoides	Kastria Cave (Greece)	Lamprinou et al. [2]
Scytonema drilosiphon	Božana Cave (Serbia)	Popović et al. [28]
Scytonema julianum	Gelda Cave (Spain), Kastria Cave (Greece), caves from Ojców National Park (Poland),	Martínez & Asencio [21], Lamprinou et al. [2], Czerwik-Marcinkowska et al. [26]
Scytonema mirabile	Božana Cave (Serbia)	Popović et al. [28]
Symphyonema cavernicolum	Gelda Cave (Spain)	Martínez & Asencio [21]
Tolypothrix epilithica	Caves from Ojców National Park (Poland)	Czerwik-Marcinkowska et al. [26]

Table 1. Cyanobacteria species reported in caves based on literature results since 2010.

photoprotective pigments and bioactive compounds. Rock inhabiting cyanobacteria can be divided into "epilithic," colonizing the substrate surface directly; "hypolithic," living under pebbles and small stones lying on the rock; and "endolithic," living in an upper layer of rock [29]. The cavernicolous cyanobacteria species reported from rock and stone wall surfaces, mostly coccoid and heterocytous types with (often colored) mucilaginous

envelopes and sheaths, tend to be dominant in terms of biomass. Cyanobacteria play a main role in the species biodiversity of caves. However, characterizing the biodiversity of caves is challenging because cyanobacterial communities often have high richness and contain numerous species that have neither been isolated nor described using traditional culturing techniques. The culture-independent methods can be applied to study cave communities and are especially powerful if combined with culture-based information. Many studies [20, 30] have described cyanobacteria occurring in both terrestrial sediments and aquatic cave environments around the world. This widespread distribution reflects the tolerance of cyanobacteria toward environmental stress due to a broad spectrum of specific properties in physiology [31]. Jones and Motyka [32] noted that a single microorganism can change from an autotroph (utilizing light for food) to a mixotroph (autotrophic microorganism that grows more rapidly in the presence of certain organic substrate) to a heterotroph (growth with no light). Most of these cavernicolous species are nonresidents transported into caves by water, air, sediment, and animals. Moreover, these enrichment-based and cultural studies have focused on typical heterotrophic microorganisms, which grow in an extreme environment [33]. Culture-independent, molecular phylogenetic techniques have since shown that many previously unknown species can be found in caves [34]. Impact of cave tourism (artificial light) is altering the natural light gradient in cave ecosystems, which may have important repercussions on the composition of cyanobacteria communities inside the caves, and that is why, lampenflora can be regarded as invasive [35]. Tourists entering limestone caves are responsible for transferring cyanobacteria spores [36], leading to unintentional biological pollution and favoring, at the same time, the colonization of other cave microorganisms [37]. As a consequence, the alteration of the natural environmental conditions in caves may also modify the cyanobacteria communities. Hobbs et al. [38] demonstrated that lampenflora does not grow at close distance from incandescent lights due to high temperature. However, the artificial illumination also influences the water content of the substrate and air. Tourist presence leads to the increase of both temperature and CO_2 concentration inside cave [15], intensifying wall erosion [39]. Cyanobacteria communities in caves are mainly composed of cavernicolous species, generally characterized by small size, resistance to desiccation, specific preferences for pH, and tolerating low nutrient levels and high conductivity. Saiz-Jimenez [40] showed that cave environment is in a constant battle to remove cyanobacteria and lint left by visitors without damaging underlying formations. Bright artificial light installed in caves for the benefits of visitors may adversely effect on drying out surfaces and decreasing relative humidity, which may be lethal to cave adapted microorganisms. Moist, humid conditions favor the growth of species on soils and rocks, for instance, *Nostoc commune* colonies which typically grow on calcareous soils or depressions on limestone surfaces. In limestone caves, cyanobacteria can be found in water bodies [41] and in subaerophytic karst habitats [42]. Cavernicolous cyanobacteria can be observed in the cave entrance illuminated by direct or indirect sunlight and caves equipped with artificial illumination, as a part of a lampenflora community around lamps [12].

Limestone caves are under immense pressure from anthropogenic activities, especially in recent years [9], and are probably one of the centers of biodiversity for certain types of cyanobacteria [2], especially for species from families Hapalosiphonaceae and Symphyonemataceae [43].

This high diversity may partly be caused by the lack of photosynthetically active radiation which is almost the only stressor in caves, whereas subaerophytic habitats are significantly affected by many stress factors such as excessive irradiance, UV, desiccation, rapid temperature change, and their combinations.

It is well known that cyanobacteria are considered the pioneering inhabitants in the caves colonization. Cyanobacteria prevail in the cave entrances compared to the other microalgae [39]; however, they colonize the various parts of the cave entrances, where biodiversity of organisms is the lowest [22]. Among many factors influencing cavernicolous species, water relations in caves are important for cyanobacteria to growth and their colonization [44]. Lamprinou et al. [4] stated that cavernicolous species are dominated by cyanobacteria, which represent the first photosynthetic colonizers on the calcareous surfaces usually thriving both as epiliths and as endoliths. Epilithic communities form extensive dark-green coverings created by *Phormidium breve* as dominant species, or pale blue-green to whitish coverings consisting *Tolypothrix epilithica*. Lamprinou et al. [30] observed predominance of Oscillatoriales group over Chroococcales, in the dim light zone, and also in the entrance, especially in speleothems exposed to light, but their presence is attributed to the chasmoendolithic mode of life.

Round [45] differentiated the distribution of microorganisms depending on the access of either natural or artificial light. Growing of cyanobacteria visible in the form of different color patches on cave walls is undoubtedly connected with the availability of light and specific limestone cave microclimate. This microclimate in caves is influenced by air circulation, hydrological conditions, and isolation of cave from the outside thermal influences [21]. Microscopic observations [21] revealed that cyanobacteria are arranged in particular communities named patinas, which are blue, brown, green, or gray. These communities contain coccoid forms that are frequently accompanied by filamentous forms that are irregularly distributed and do not present stratification. Generally, there are two different areas of the caves. One area is the entrance, where the microclimate is influenced by the light, temperature, and relative humidity fluctuating throughout the year. Patina is greenish-bluish formed by coccoid species only, and there are also grayish patina constituted by coccoid and filamentous species. The second area is the inside with a stable temperature and relative humidity and very low light. The patina found there are greenish-bluish formed by only coccoid species, brownish-gray patina constituted by coccoid forms and filamentous forms, and bluish-grayish patina formed by coccoid forms and filamentous forms. On the other hand, the cave tourism is an important factor causing increase of temperature and environmental changes. Pouličková and Hašler [11] observed the majority caves in Europe are characterized by an average humidity (circ. 70%), and their entrance walls usually are covered by cyanobacteria. The development of cave tourism requires alteration of natural corridors, installation of lighting, pathways, platforms, and associated infrastructure [46]. On the other hand, caves impacted by severe disturbances, including tourism and artificial illumination, have never been completely restored to their former ecological state [47]. Under such conditions, the oligotrophic nature of cave environments is expected to change through organic inputs that alter both the food web and the abundance and distribution of cave organisms [40].

Pentecost and Zhang [48] and Uher and Kovacik [49] noted that type of substratum is an important factor determining the species composition, distribution, and structure of cavernicolous species. They observed that growth of cyanobacteria such as *Anabaena oscillarioides*, *Gloeocapsa biformis,* and *Nostoc punctiforme* was dependent on the temperature, light, and humidity. These cyanobacteria prefer the humid places during their development, but they also display a considerable resistance to drying as well as to a low air temperature during winter. The adaptation mechanism of cyanobacteria living in a low temperature is not yet precisely known [39], but *Scytonema julianum* is reported as an atmophytic cyanobacterium grown in limestone cave walls in small clusters in shaded vadose settings throughout the world and is prone to rapid calcification [50].

The cyanobacteria species distribution in relation to cave morphology, lithic substrate, and microclimatic conditions still remain a challenge for further studies.

4. Conclusions

An investigation of the diversity and ecology of cyanobacteria in limestone caves has been conducted for many years. Cyanobacteria were the dominant group of phototrophs colonizing cave walls. Chroococcales was the most common cyanobacterial order (with *Gloeocapsa* as the most frequently encountered cyanobacterial genus), followed by Nostocales. The most widespread and abundant species were *Aphanocapsa muscicola, Gloeocapsa biformis,* and *Nostoc commune.* Caves impacted by severe disturbances, including tourism and artificial illumination, were never been completely restored to their former ecological state [47]. Principally, every visitor entering a cave, from the professional speleologist to tourists, has the potential to exert a negative impact on the cave ecosystem.

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Section 3

Cultivation and Adaptations

Chapter 4

Cultivating *Spirulina maxima*: Innovative Approaches

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Additional information is available at the end of the chapter

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Abstract

This chapter reports an annual production of *Spirulina* (*Arthrospira*) *maxima* in Ansan, South Korea (37.287°N, 126.833°E) with temperate four seasons climate for testing industrial application. Construction on pilot plant of semi-open raceway system (ORS) with each 20 ton culture volume has been established in early 2011 based on building information modeling (BIM). An optimized design of pilot culture system for microalgae scale-up culture in temperate area and details of culture was presented. In scale-up trials using two ORSs, the strain displayed satisfactory annual growth under batch condition. In an annual trial, average biomass concentration was recorded at 0.99 ± 0.16 g/L, which showed stable productivity in a year. Maximum concentration was estimated at 1.418 ± 0.09 g/L in August, while minimum production was estimated at 0.597 ± 0.05 g/L in October. Despite insufficient solar radiation and nutrients, ORS was favorable for *S. maxima* production. The technical strategies contribute to the annual production of *S. maxima* in this region: controlling the culture temperature, reducing production cost, and retrospective climatic data-based BIM construction of the greenhouse. Consequently, pilot production of *S. maxima* was feasible in Korean climates, a region previously thought to be outside its geographic limits.

Keywords: *Spirulina* production, temperate climate, BIM-based pilot plant construction, Korea

1. Introduction

Spirulina (*Arthrospira*) is a 3.6-billion-year-old cyanobacterium and inhabits alkaline aquatic ecosystems from freshwater to seawater [1, 2]. As sustainable primary producers, the cyanobacteria have been studied on various aspects centered by bio-industry and environmental

bioremediation [3–6]. *Spirulina* that highly contains various functional materials has been focused as an important nutrition source for the future of humans and animal feeds, so that related industry has been gradually growing [2, 7, 8, 12]. In addition, the biomass plays an important role in broad ranges of industries [9, 10] and functional foods using proteins [3, 11]. In particular, *S. platensis* has been mostly studied for industrial production, in which the most important factors for an increase of production could be nutrient concentration, light intensity, and optimal water temperature [12]. *Spirulina* sp. including other microalgae are cultured on a large scale worldwide for industrial use [13–17]. Proteins and phytopigments (phycocyanin and β-carotene) along with polyunsaturated fatty acids from *Spirulina* are one of the high value-added materials that bring health to humans [2, 13, 18].

Nowadays, microalgae are mostly cultured in photobioreactors (PBRs) and open raceway systems (ORSs) for industrial production [2, 19, 20]. Advantages of PBR include applicability of various designs in production, easy control of growth condition, prevention of biological contamination, and high productivity. On the contrary, it requires high expenses in initial investments, device maintenance, and expansion of mass production facility [20–22]. In contrast, ORS can directly use solar energy and CO_2 in the air though it has a low aerial productivity than PBR, and it is also advantageous due to inexpensive materials for facility (PVC, FRP, concrete, plastic, and soil) and easy to scale up structure. In respect of commercialization, ORS needs a low initial investment cost, while it has a highly efficient productivity, so that ORS has been attracting more interest [15, 20, 23–30]. However, there are still steps that need to be taken for the commercialization of ORS, which includes a control of water temperature and light intensity depending on season, elevation of aerial productivity, development of highly efficient microalgae species, development of low cost and highly efficient culture medium, technology of contamination improvement, and establishment of protocol for year-round culture and harvest [19, 20, 31–33].

Culture conditions of microalgae in ORS and designing of system are closely related with environment of selected area. *Spirulina* was the most widely used in outdoor cultivation trials and interests for commercial production have been increasing in many places based on the studies of intensive ecological and physiological research and development over four decades. However, this cyanobacterium needs high temperatures for optimal growth, thus commercial production has been limited until subtropical areas. In temperate regions, high temperatures are recorded in summer season, while the temperature is certainly low in fall and winter seasons. The overall temperature range should not be a suitable range for *Spirulina* growth in a year. Consequently, little information of commercial production has been reported in temperate areas [32, 34, 35]. Thus, an environmental analysis needs to be carried out on selected area for a culture system with maximal productivity based on which culture system needs to be constructed. Recently, the concept of building information modeling (BIM) developed in construction field was first introduced by the National Institute of Standards and Technology (NIST) of the Department of Commerce in the USA in 2007. BIM refers to a recent construction process that analyzes data with 3D modeling method using big data and information of the past and then applies it from designing to installation process, avoiding existing 2-D design. Therefore, BIM technology is advantageous due to cost saving for installation operation and prediction of future [36–38]. As shown in success cases such as the Disney Concert Hall of the US, the Olympic Main Stadium of Beijing, and Melbourne Recital Center, BIM technology has emerged as an important issue in architectural industry, so that it is considered beneficial to apply it to a microalgae production facility.

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From 2008, small-scale (laboratory to 1.5 ton) experiments have been conducted to investigate the biomass production combined with culture conditions and low-cost medium of *S. platensis* and *Spirulina maxima* in Korea [33, 39, 40], but further progress was not reported. In 2011, the first pilot-scale *Spirulina* plant with semi-open raceway ponds was established in Ansan [41]. The purpose of this chapter is to present for construction technology of BIM-based ORS allowing year-round culture in the environment with four seasons, so that an ORS was constructed in a glass greenhouse structure and a culture study was performed on a pilot scale (173.5 m^2). In addition, in order to verify sustainability of the system, year-round biochemical quality analyses on year-round batch culture of *S. maxima* that has a characteristic of strong alkaline culture and on produced-dried biomass were performed at the same time.

2. System construction and strategies for culture plan

2.1. The production site

The city of Ansan was selected for the pilot study for production of *Spirulina*. Ansan is located in Western South Korea (37.287°N, 126.833°E), about 30 km south of Seoul, on the coast of the Yellow Sea. Recent average temperatures generally exceed 30°C in summer, and in winter are above −5°C. The average temperature is 12.5°C, and number of sunny days per year generally ranged from 86 to 124, with >10 h of sun per day for the whole year. Mean monthly temperatures are shown in **Figure 1A**. **Figure 1B** shows that weekly average solar radiation ranged from 1721 to 5671 Wh/m^2. From 2006 to 2010, the number of rainy days per year ranged from 99 to 138, and average annual rainfall was about 1150 mm in the locality of the cultivation area.

2.2. Construction of culture system and its structure

Figure 2 shows schematic processes for planning and construction of *Spirulina* culture system using BIM technology. A pilot system for microalgae production was constructed in the Korea Institute of Ocean Science and Technology based on BIM technology which analyzes atmospheric environment data (temperature, solar radiation, and shadow effect etc., **Figure 3**) of the past for a long time, and predicts the future based on the data in order to design an optimal eco-friendly structure.

Figure 4A is a vertical section of microalgae pilot system constructed based on BIM, in which the roof and side windows were designed with a maximal consideration of natural ventilation, and optimal construction cost and efficiency was realized by a four-way slide window at the side and the introduction of automatic opening and shutting system on the roof. **Figure 4B** is a horizontal section of the modified ORS culture facility. Each size of the culture facility was 10,000 (W) × 3250 (L) × 550 (H) mm, and the culture raceway was finished with concrete after vertical excavation of the ground as deep as 600 mm for the purpose of using geothermal heat as shown in **Figure 4**. Depth of medium for the culture was maintained as 400 mm. Boiler pipes were buried in the concrete floor of the modified ORS for maintenance of culture temperature in the winter.

Computational fluid dynamics (CFD) was conducted to analyze and optimize the mixing (e.g., water flow and paddle rotational speed) of medium with *S. maxima* cells in the ORSs. The main purpose is to find the suitable rotational speed of the paddle to maximize mixing in the flow field.

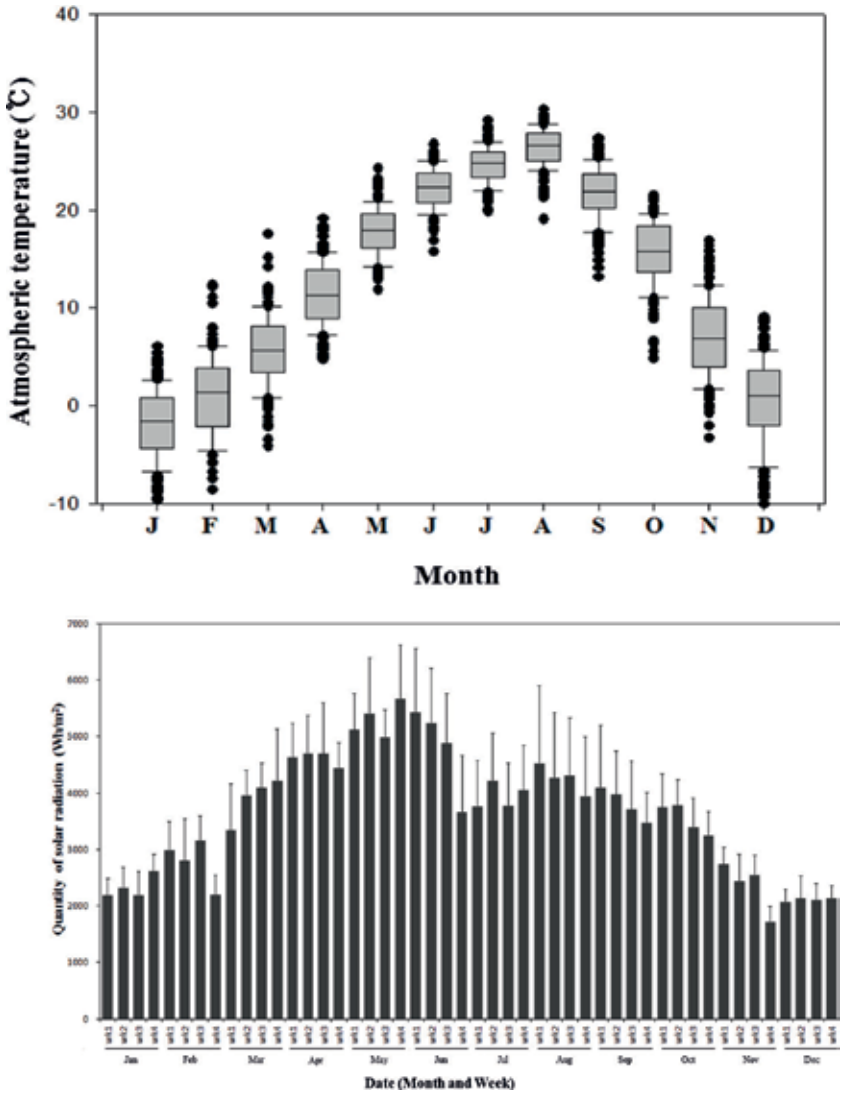

Figure 1. Mean monthly temperature (A) and integrated weekly solar radiation (Wh/m^2) (B) at the location of the study for past 5 years during 2006–2010.

The commercial software Flow-3D (version 10.0) was chosen for this analysis. The simulation was performed by ARA Consulting & Technology Co., by applying two rotational speeds (15 and 30 rpm) (**Figure 5**). As a result, rotational speed was selected as 15 rpm in the ORSs. Simplified structures of the ORS and the greenhouse for *S. maxima* cultivation were shown in **Figure 6**.

2.3. The microorganism, experimental design, and culture conditions

The axenic culture of *S. maxima* Cy-23, was obtained from Korea Marine Microalgae Culture Center (Pukyong National University, Busan, Korea) and was initially inoculated in a 5 L conical flask containing modified *Spirulina* medium [42] at plant room temperature (23–30°C). Thereafter, secondary culture with scale up was performed in a 300-L PE circular cylinder

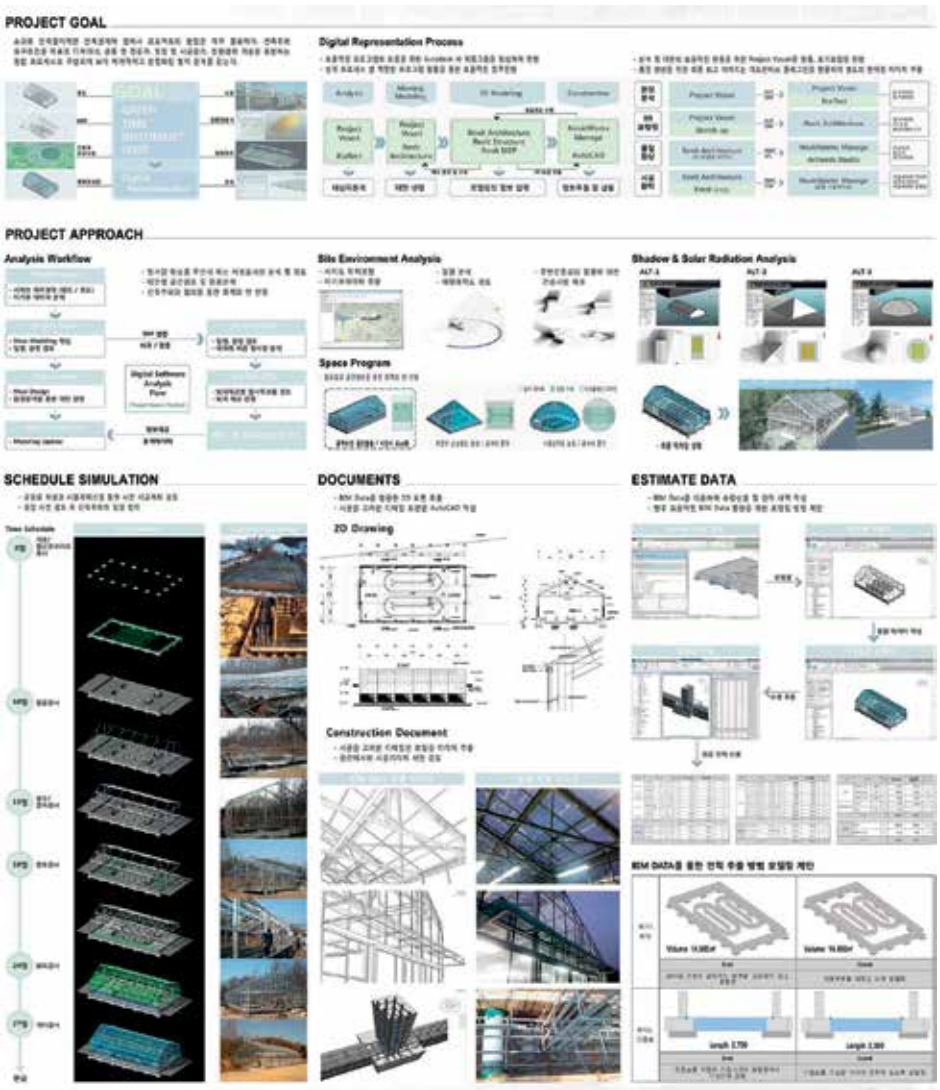

Figure 2. Schematic processes for planning and construction of *Spirulina* culture system using BIM technology.

raceway on the 10th day when the culture concentration of *S. maxima* reached to 0.5–0.8 g/L. Once concentration of secondary culture reached to 0.5–0.8 g/L on the 17th day, 20 tons of each culture medium with the same composition was made and added to the modified ORS. After 5 h, 300 L of sample from the secondary culture was inoculated. The rotational speed of the paddle wheel that circulates and mixes the culture medium was kept at 15 rpm. Batch culture was performed in the ORS for about 1 year from April 4, 2011 to March 16, 2012.

2.4. Maintaining culture system

The electric boiler that was installed for maintenance of temperature in the winter was operated from late October to early April to heat the system electrically and to maintain optimal

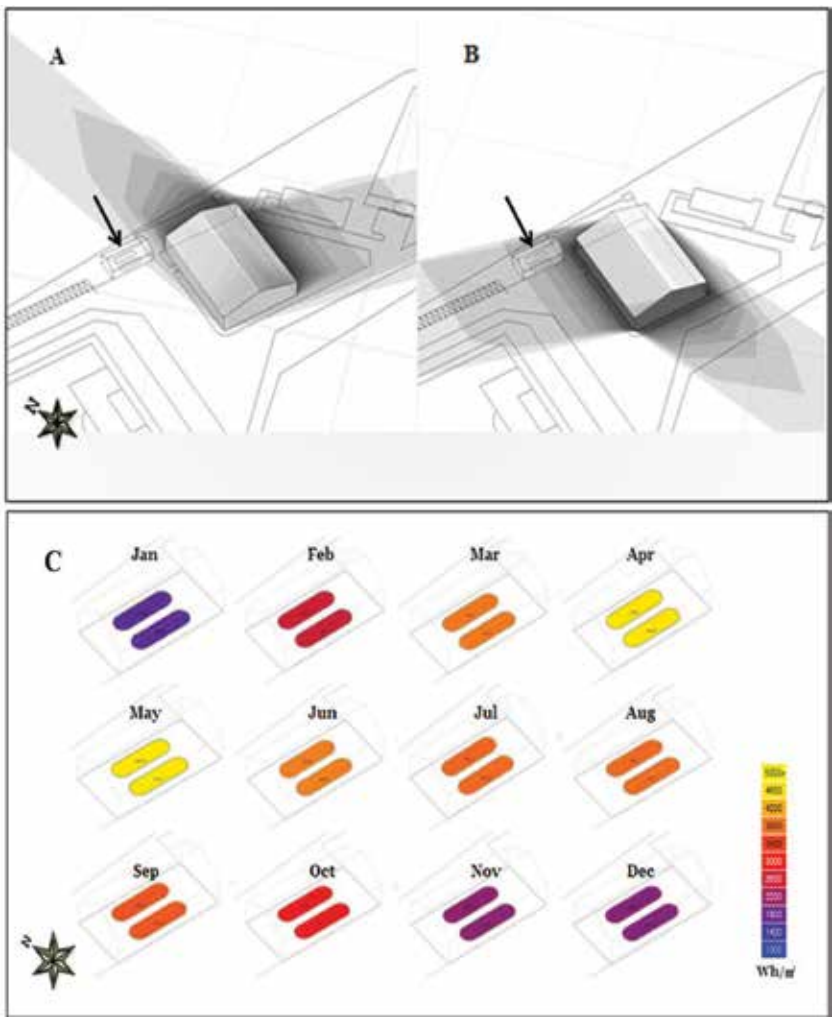

Figure 3. The retrospective analysis of BIM based 3D modeling for visualization of shadow area at summer (A) and winter (B) solstices and estimation of monthly solar radiation of two raceway pond (C) at the location of the study for past 5 years during 2006–2010. Arrows indicates the location of culture area.

water temperature. The temperature of the culture medium was maintained between 20 and 25°C, and the water temperature for October when its operation was initially started was in the range of 21–23°C. Total electricity consumption during the period of boiler operation was measured by using an electronic watt-hour meter (LD3410CT-005Te). The boiler operation was set as a variable type depending on variation in water temperature (on 30 min, off 30 min), and the maximum operation rate (on 50 min, off 10 min) was used to minimize fluctuation of water temperature during the period of rapid drop in room temperature in the winter. Daily measurement values were summed every week and expressed in mean value ± standard deviation for an analysis of each environment and electricity consumption data.

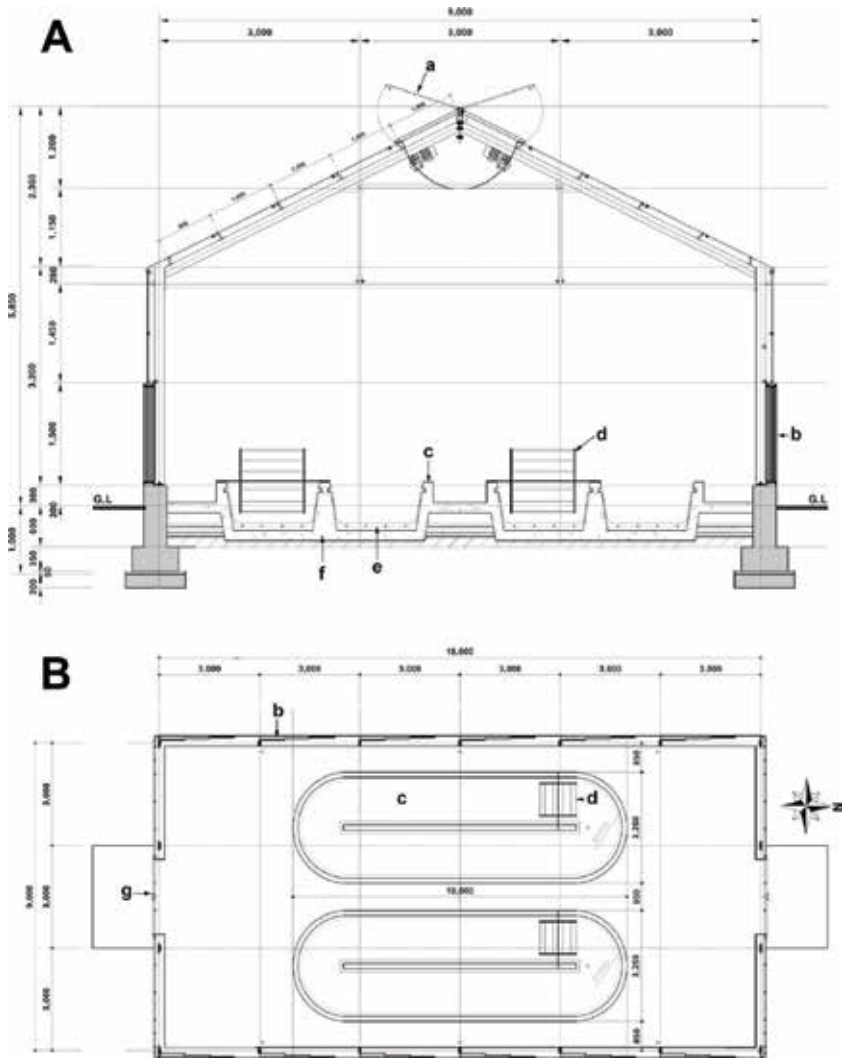

Figure 4. A cross sectional view of the designed open raceway system for pilot production of *Spirulina maxima* using BIM analysis (patent no. 10–1,142,358, 10–1,142,359, 10–1,110,068). A: Vertical view of the system; a: Automatically opening and shutting window, b: 4-way sliding windows including anti-insect nets, c: Raceway pond, d: Paddle-wheel, e: Electronic boiler pipe, f: Concrete structure. B: Horizontal view of the system; main gate (g) has located to the south.

2.5. Biomass concentration, harvesting, and productivity

Before harvesting, the biomass concentration was estimated by sampling and filtering 20 mL of each culture using a vacuum pump and GF/C filter paper (Whatman), and the filter paper was dried in a dry oven (65°C) for 24 h, followed by the measurement of biomass. Biomass was measured two times a week. For harvesting, cultured *S. maxima* samples in 3 tons of culture medium were harvested by using a Tubular Separator (GQLY series) at 15,000 rpm for 3 h in an interval of a week. Harvested samples were kept in a −50°C deep freezer (DEASAN) for a biochemical analysis and then freeze-dried by using a freeze dryer (ILSHIN).

Areal productivity was calculated by using the following equation. Data used for analysis were monthly mean value (mean value ± standard deviation) by adding the weekly measurement values.

$$\text{Areal productivity (AP, g/m}^2\text{/d)} = \text{Volumetric productivity (VP)} \times (\text{Odepth} \times 1000) \quad (1)$$

where Odepth is the pond operating depth.

2.6. Analysis of biochemical components and pigments

The crude biochemical composition of cultured *S. maxima* was determined according to the AOAC method [43]. Crude carbohydrate was determined by the phenol–sulfuric acid reaction, crude lipid was extracted by the Soxhlet method, and crude ash was prepared at 550°C

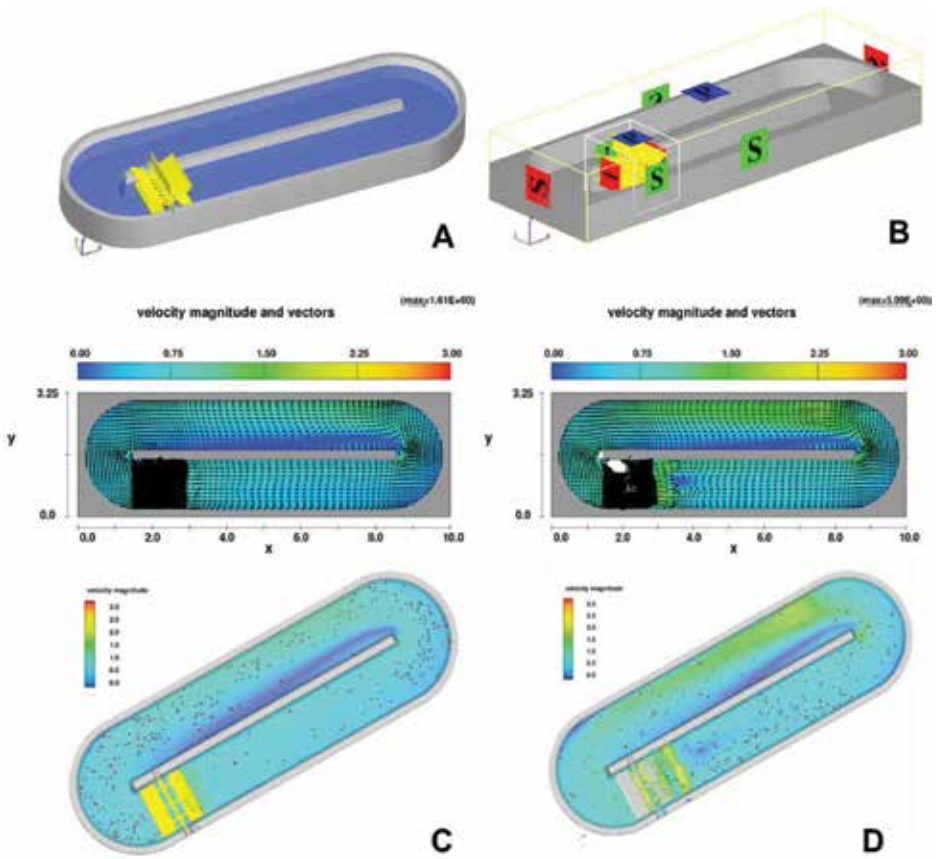

Figure 5. Summarized results on mixing simulation of medium with *S. maxima* cells in the ORSs by applying CFD of flow-3D software. A: 3D applied raceway pond (10 m in length, 3.25 m in width and 0.6 m in depth) with paddle, B: Interface condition of boundary line by interactive boundary layer modeling techniques, C and D: Velocity magnitude and particles maps at 60 second after operation for various aspect of particles with different rotational speeds (C: 15 rpm, D: 30 rpm).

Figure 6. Simplified inner structure of ORS and the greenhouse for *S. maxima* cultivation at Ansan City, KIOST. The number of 20 was harvesting time and the number of 167 was the days after inoculation. Photographed by Taeho Kim in 2011.

in the dry-type furnace. The moisture was determined by keeping the sample in a dry oven at 105°C for 24 h and the crude protein was determined by the Kjeldahl method. Unfortunately, the samples of November and December 2011 were denatured by sudden trouble of refrigerator for biochemical and pigments analysis.

For phycocyanin analysis, dried *S. maxima* powder was weighted accurately to 50 mg, and then mixed with 10 mL sodium phosphate buffer (pH 7.0) in centrifuge tube. The mixed sample was sonicated for 1 min and stored in refrigerator overnight maintained at 20°C. The sample was mixed well using vortex and then was centrifuged for 20 minutes at 10°C at 3000 rpm. The supernatants were repeatedly collected until the color came off. After collecting all the phycocyanin, the supernatant was filtered using GF/C filter, and the red absorbency of each replicate was at 620 and 652 nm using phosphate blank buffer with spectrophotometer (Optizen POP bio). Concentration of phycocyanin was then calculated using below formula [44].

$$\mathrm{C-PC\,(mg/L)} = \left(A_{620} - 0.474 \times A_{652}\right)/5.34 \tag{2}$$

$$\text{phycocyanin}\,(\mathrm{mg/g}) = \frac{\mathrm{C-PC}\left(\frac{\mathrm{mg}}{\mathrm{L}}\right) \times \text{buffer volume (mL)}}{\text{sample weight (g)}} \times \frac{1\ \mathrm{L}}{1000\ \mathrm{mL}} \tag{3}$$

For chlorophyll-*a* analysis, dried *A. maxima* powder was weighted accurately at 50 mg and then mixed with 5 mL-90% acetone. The mixed sample was sonicated for 1 min and then stored in refrigerator for 30 min maintained at 4°C. The pigment extracts have to be kept on ice and in the dark. The sample was mixed well using vortex and then was centrifuged

for 20 minutes at 10°C at 3000 RPM. The supernatants were repeatedly collected until the color came off. After collecting all the chlorophyll, the supernatant was filtered using a 0.2 μm syringe filter, and the absorbency of each replicate was at 625, 647, and 664 nm using phosphate blank buffer with spectrophotometer (Optizen POP bio). Concentration of chlorophyll was then calculated using below formula [45].

$$C_a(\text{mg/L}) = (12.62 \times A_{664}) - (2.99 \times A_{647}) - (0.04 \times A_{625}) \quad (4)$$

$$\text{Chlorophyll} - a\ (\text{mg/g}) = \frac{C_a\left(\frac{\text{mg}}{\text{L}}\right) \times \text{acetone volume (mL)}}{\text{sample weight (g)}} \times \frac{1\ \text{L}}{1000\ \text{mL}} \quad (5)$$

2.7. Measurement of climatic and culture conditions

Various parameters of the system were measured on a daily basis including room temperature (TENMARS) and light intensity (Lux Meter TM-205), water temperature (UNIS thermometer), pH (pH METER D-51, HORIBA), and salinity (SALT MATER YK-31SA). Although humidity was not a variable of interest, it seems the humidity was dropping in the plant as the level of medium kept in the plant was consistently lowering by evaporation. Evaporation amount was measured during August when culture medium was highly evaporated. To specify the amount of water being evaporated each day per unit area (m^2), daily evaporation rate was measured and then averaged as ml/m^2/h. The amount of evaporation was then supplemented daily with underground tap water in KIOST ($HCO_3$4 6.4 mg/L, Ca 20 mg/L, Cl 13.6 mg/L, SO_4^{2-} 11.4 mg/L, Na 8.64 mg/L, Mg 3.99 mg/L, K 1.97 mg/L, T-N 1.66 mg/L, NO_3-N 1.61 mg/L, T-P 0.02 mg/L, Co, Mo, and B 0 mg/L while Fe, Zn, Cu and Mn were not detected, and pH was 7.3).

A statistical program (IBM SPSS, NY, USA) was used for statistical analysis in order to test significance of environmental factors and pigments and biomass of *S. maxima* (one way ANOVA, Tukey test, *P* values <0.05). Monthly variations among climatic and culture conditions, biomass, biochemical components, and pigment concentrations were statistically analyzed by one way ANOVA with Pearson's multiple range tests at $p < 0.05$ (SPSS version 12.0, NY, USA) for the identification of significant seasonal differences during the study period. All analyses were performed on triplicate samples.

3. A feasibility of *Spirulina* annual production in the area

Disadvantages for *Spirulina* production in this area are not only the average temperature (12.5°C), but putative aerial contamination of the medium by the dusts and microorganisms from reclaimed land in the City. The period of cultivation would be approximately <200 days for growing in open raceway ponds in this region without trials to control the disadvantages. In order to overcome the problems, the most important approach in annual cultivation in this area was to construct greenhouse over the raceway ponds. The greenhouse was made of a framework (mixture of steel and cement) and transparent glasses. The glass greenhouse enhances the culture temperature significantly (**Table 1**) and the growth period prolonged during a year.

The maximum daily medium temperature of the culture was over 20°C and up to 30°C during the period from January to August. **Table 1** and **Figure 7** present year-round change data of radiation amount (μmol/m^2/s), temperature of culture medium, room temperature, salinity, pH, and electricity consumption during the culture of *S. maxima* from April 2011 to March 2012. As for illumination intensity during the study period, the maximum value was 1590 μmol/m^2/s, and the minimum value was 7 μmol/m^2/s. Year-round water temperature of culture raceway for *S. maxima* was between 16.0 and 33.0°C, and mean water temperature was 23.6 ± 3.2°C.

Temperature of culture medium showed a change in a range of 20.2–26.8°C from April 4, 2011 to May 31. The highest water temperature of the year was 33.0°C on July 26 as an effect of increase in outer temperature. In addition, the average evaporation rate in August 2011 was 701 ± 136.4 ml/m^2/h. Water temperature gradually decreased from October, went below 20°C during the second week of December and recorded the lowest water temperature of the year at 16.0°C on February 23, 2012. Culture medium temperature for optimal growth of *Spirulina* was between 35 and 37°C, and should be kept at least at 15–20°C [2]. Since severe air temperature in this area was recorded from −18 to 36°C, and it was below 10°C on average in the winter, the ORS with glass greenhouse was electrically heated by the boiler during the period from late October to early April to maintain at least 15°C, the lowest temperature for *Spirulina* culture. These results should serve as fundamental data for setting temperature and running a boiler in order to maintain optimal medium temperature in the winter.

The mean initial salinity of May and June was 16.6 ± 0.9 psu due to the effect of added tap water. As culture days increase, salinity concentration showed a range of change between 13.1 and 18.4 psu during the year due to effects of evaporation of culture medium and supplementation of freshwater. In addition, the mean salinity concentration during the entire culture period was 16.5 ± 1.3 psu. The pH change during the culture period showed a relatively small variation between 9.9 and 11.9. Variation of pH from May 4, 2011 to September 9 was between 9.9 and 10.97, pH change between September 14 and September 30 was ranging between 11.15 and 11.90, and then it went down to below 11.0 from October 4. The ending pH on March 16 was 9.97. Internal room temperature of the plant during the culture period was in the range of 3.2–55.0°C, and the mean room temperature was 24.3 ± 10.5°C. The mean total electricity consumption (kWh) of the microalgae pilot plant was 10.3 ± 1.1 kWh per day during the initial culture between April 4, 2011 and May 4, during which the boiler was operated for maintenance of optimal medium temperature (20.2–26.1°C). Boiler operation was stopped between May 6, 2011 and October 16, 2011 for optimal temperature, during which 1.3 ± 0.6 kWh of electricity was used on average per day.

The range of biomass of *S. maxima* that was produced year-round using batch culture method in the raceway system with glass greenhouse structure in the present study was 5.68–37.67 g/m^2/d, in which the highest productivity of the year was recorded as 37.67 g/m^2/d in the summer when temperature increased. According to a study on *S. platensis* culture in a raceway system (750 L) by Richmond et al. [46], productivity was 15–27 g/m^2/d, whereas a study on culture of *S. platensis* in a raceway system (135,000 L) conducted in Spain showed 2–17 g/m^2/d [47]. Considering that limiting factors important for growth and productivity of microalgae are solar radiation, carbon supply, water temperature, and dissolved oxygen [5, 46, 48], a control system on these factors are also critical. BIM designing technology that was introduced in the present study to predict these effects had advantages including investigation of spatiotemporal

	2011									2012		
	Apr	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**
WT	22.4 ± 1.3	23.3 ± 1.7	25.2 ± 1.9	27.7 ± 2.4	29.0 ± 1.8	25.6 ± 1.9	21.2 ± 1.8	23.9 ± 2.4	21.3 ± 1.8	19.5 ± 1.4	20.4 ± 2.2	22.6 ± 1.6
RT	29.3 ± 4.1	26.4 ± 4.1	28.1 ± 3.8	34.8 ± 8.0	37.0 ± 4.6	35.9 ± 6.2	28.2 ± 3.5	22.9 ± 4.8	14.0 ± 3.6	10.8 ± 4.5	10.8 ± 4.8	15.7 ± 3.9
SR	822.2 ± 460.2	480.4 ± 397.6	519.4 ± 391.4	219.4 ± 179.4	351.0 ± 318.6	604.4 ± 377.1	382.2 ± 330.2	354.2 ± 351.8	328.0 ± 214.7	336.4 ± 310.8	371.8 ± 344.7	317.4 ± 404.6
Salinity	16.2 ± 0.1	15.6 ± 1.3	16.5 ± 0.8	18.1 ± 0.7	16.5 ± 1.6	16.3 ± 0.8	17.0 ± 0.4	16.2 ± 0.9	17.5 ± 1.3	16.4 ± 1.4	15.3 ± 1.9	13.5 ± 0.9
pH	9.69 ± 0.2	10.4 ± 0.1	10.3 ± 0.2	10.4 ± 0.3	10.4 ± 0.3	11.1 ± 0.4	10.5 ± 0.1	10.5 ± 0.1	10.6 ± 0.3	10.3 ± 0.3	10.2 ± 0.2	10.0 ± 0.1
TEC	290.6	72.5	42.0	38.9	39.1	38.5	144.4	331.6	475.3	453.2	440.6	389.9

WT: water temperature (°C); RT: room temperature (°C); SR: solar radiation (μmol/m²/s); TEC: total energy consumption (kWh).

Table 1. Monthly average data of water temperature (WT), room temperature (RT), solar radiation (SR), salinity, pH, and total energy consumption in the microalgae pilot plant.

http://dx.doi.org/10.5772/intechopen.74265

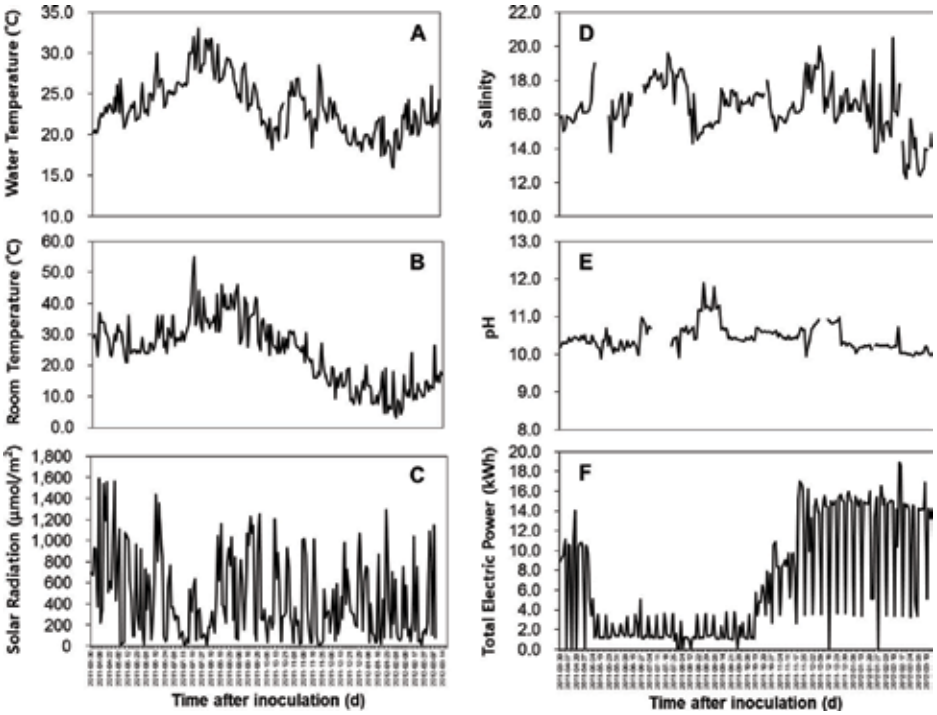

Figure 7. Annual variations of daily data on medium water temperature (A), room temperature (B), solar radiation (C), salinity (D), pH (E) and use of total electric power (F) in the *S. maxima* culture pond during the period from April 4, 2011 to March 16, 2012.

relevance of construction using 3D modeling, and prediction of problematic factors in advance including an analysis of environmental interference as known already [36–38], which resulted in desirable *Spiruina* biomass produced from the facility that designed based on an environmental analysis (atmospheric temperature and radiation amount) known as major factors of *S. maxima* growth, and predictions of environmental interference (e.g., shadow effect in **Figure 3**). **Table 2** and **Figure 8** show year-round biomass of *S. maxima* during the culture between April 8, 2011 and March 15, 2012.Variations of biomass and daily productivity were 0.227–1.507 g/L and 14.2 ± 9.6–31.42 ± 4.8 g/m^2/d, respectively, during the culture period, and mean values were 0.96 ± 0.24 g/L and 24.2 ± 5.90 g/m^2/d, respectively. The mean daily productivity of April when culture was started was 14.2 ± 8.1 g/m^2/d. The mean daily productivity gradually increased from May reaching to 23.92 ± 2.98 g/m^2/d. Thereafter, there were changes in productivity due to harvest of *S. maxima* for component analysis and supplementation of freshwater for evaporation of culture medium. The highest productivity of the year (31.42 ± 4.8 g/m^2/d) was achieved in August when temperature of culture water was high, which was statistically significant ($p < 0.05$), and the lowest productivity of the year (18.81 ± 4.3 g/m^2/d) was recorded in October. When comparing the results of this study with most preceeding studies, the raceway system with a glass greenhouse structure in the present chapter achieved the maximum aerial productivity (**Table 3**). In addition, despite the continuous study in batch culture method without resupply of nutrients, a high aerial productivity (24.2 g/m^2/d) in annual average was obtained.

	2011									2012		
	Apr	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**
Biomass (g/L)	0.57 ± 0.4	0.96 ± 0.2	1.02 ± 0.1	1.19 ± 0.2	1.26 ± 0.2	0.97 ± 0.1	0.75 ± 0.2	0.88 ± 0.2	1.01 ± 0.1	0.96 ± 0.1	0.96 ± 0.1	0.95 ± 0.2
Productivity (g/ m^2/d)	14.2 ± 9.6	23.92 ± 5.5	25.38 ± 3.5	29.65 ± 3.8	31.42 ± 4.8	24.26 ± 3.1	18.81 ± 4.3	21.95 ± 3.9	25.20 ± 3.3	24.09 ± 3.0	23.97 ± 3.3	23.65 ± 5.2

n.d.: not determined.

Table 2. Monthly average data of biomass and productivity of ***S. maxima*** in the microalgae pilot plant.

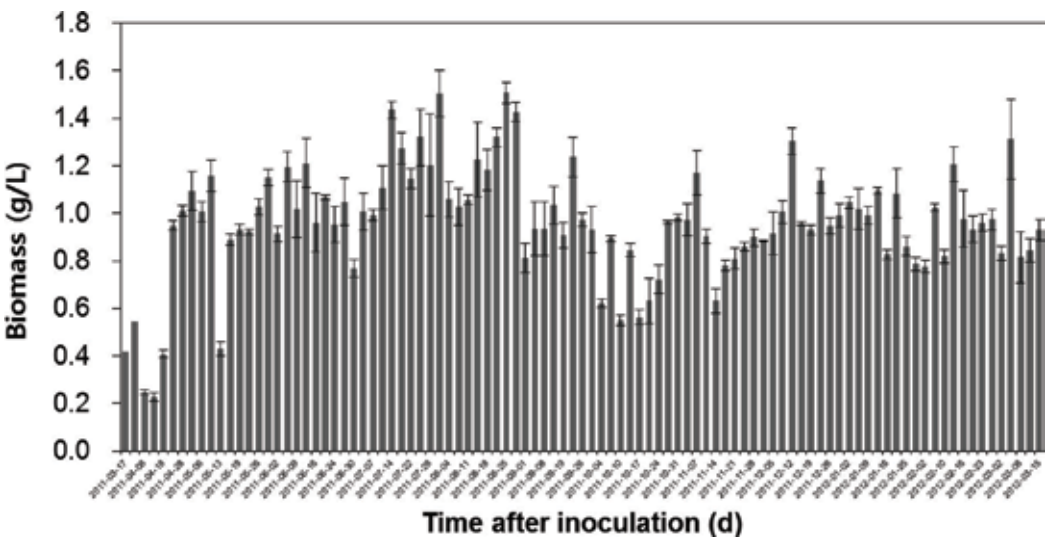

Figure 8. Annual variation on the biomass concentration of two times weekly-harvested *S. maxima* during a culture period.

Thus, it has confirmed a foundation to use a raceway system with a glass greenhouse structure or photobioreactor for countries with four distinct seasons.

Table 4 presents year-round ratios of protein, carbohydrate (CHO), and lipid contents in *S. maxima*. Protein content of *S. maxima* in April 2011 when culture was started was 40.08%, which gradually increased to 47.64% in July, the highest. As culture period became longer, ratio of protein gradually decreased and recorded 23.71% in February 2012, the lowest of the year. Protein content was higher from the spring to the early fall compared to other components, which had significant correlations with temperature of culture medium and solar radiation ($p < 0.05$). Ratio of CHO was 36.81% in April 2011 when culture was started, which decreased to 20.06% in June. Ratio of CHO highly increased from September 2011, and reached a peak of 42.19% in January in 2012, which was contrasted to the content of protein. Thus, protein and CHO contents exhibited a significant inverse correlation depending on season ($p < 0.05$, $r^2 = 0.8542$). Similar to the results of preceding studies, high protein contents were found in *S. maxima*, and changes in protein and CHO depending on season, and changes in

Cultivation system	Culture volume (L)	Productivity ($g/m^2/d$)	Species	Location	References
Raceway	600	5–40	*Tetraselmis* sp.	Japan	Matsumoto et al. [63]
Raceway	—	1.6–3.5	*Dunaliella salina*	Spain	Garcia et al. [64]
Raceway	110	20–37	*Dunaliella salina*	Perth, Australia	Moheimani and Borowitzka [29]
Raceway	750	15–27	*Spirulina platensis*	Israel	Richmond et al. [46]
Raceway	—	8.2	*Spirulina platensis*	USA (California)	Belay [65]
Raceway	282	14.47 ± 0.16	*Spirulina platensis*	Italy	Pushparaj et al. [25]
Raceway	135,000	2–17	*Spirulina* sp.	Spain	Jimenez et al. [15, 37]
Raceway	—	9–13	*Spirulina* sp.	Mexico	Olguin et al. [66]
Raceway	500	11.2	*Tetraselmis* sp.	Japan	Matsumoto et al. [67]
Raceway	300–600	5–26	*Tetraselmis suecica*	Italy	Pedroni et al. [68]
Inclined thin layer pond	1000	10–30	*Chlorella* sp.	Czech Republic and Spain	Doucha and Livansky [69]
Inclined thin layer pond	~2500	19	*Scenedesmus obliquus*	Rupite, Bulgaria	Dilov et al. [70]
Circular central pivot pond	1960	1.61–16.47	*Chlorella* sp.	Japan	Kanazaqa et al. [71]
Open culture system	2400–16,200	19–22	*Chlorella* sp.	China	Tsukuda et al. [72]
Semi-open raceway	10,000–15,000	5.68–37.67	*Spirulina maxima*	Ansan, South Korea	In this study

Table 3. Comparison of biomass productivities of various microalgal species in outdoor open pond culture (modified from Borowitzka et al. [61]).

pigment contents were also identified [49–53]. Markou et al. [54] reported that although CHO content of *S. platensis* was between 10 and 20% in general, the limitation of phosphorus components in nutrition source resulted in an increase to 60–65%. In addition, Markou et al. [55] reported that a control of medium components for *S. platensis* caused elevation of CHO content among general components in a study on conversion of microalgae components to bioethanol. CHO content in the present study on culture of *S. maxima* showed a year-round change between 20.06–51.37%, and significantly increased at the later stage of culture. Since the present study performed a year-round experiment in batch–culture method, it seems that nutrition sources including phosphorus component was limited at the later stage, which might have caused elevation of CHO content and reduction of protein content. Batista et al. [56] reported protein content of *S. maxima* as 44.9 ± 1.8%, and Usharani et al. [57] reported protein content of *Spirulina* as 55–70%. Protein content in the present study was 40.08% in the beginning of culture and 23.71–47.64% in year-round content. Protein contents of *S. maxima* in year-round culture were the highest in July 2011 and the lowest in January 2012. In addition, protein content was significantly correlated with medium temperature and solar radiation. Jacob-Lopes et al. [58] reported that the change of light cycles (day/night) was closely related with microalgae production, and production decreased as the condition of darkness continued. Protein

	2011									2012		
	Apr	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**
Protein (%)	40.08	37.77	36.98	47.64	46.48	40.62	32.26	n.d.	n.d.	25.19	23.71	31.10
CHO (%)	36.81	28.04	20.06	25.16	28.52	37.47	47.35	n.d.	n.d.	51.37	42.19	42.92
Lipid (%)	7.16	20.68	11.03	9.13	8.01	7.29	5.90	n.d.	n.d.	4.43	5.77	8.17
Ash (%)	10.30	10.64	24.66	14.24	13.42	10.44	9.03	n.d.	n.d.	11.31	24.24	12.98
Moisture (%)	5.66	2.87	7.27	3.83	3.56	4.18	5.47	n.d.	n.d.	7.70	4.09	4.82
Chlorophyll a (mg/g)	6.1 ± 0.1	4.2 ± 0.1	3.3 ± 1.6	5.6 ± 1.8	6.3 ± 2.0	3.9 ± 0.6	3.0 ± 0.1	n.d.	n.d.	1.7 ± 0.1	1.8 ± 0.6	2.7 ± 0.1
Phycocyanin (mg/g)	28.5 ± 0.9	28.7 ± 8.5	14.8 ± 3.1	53.5 ± 11.6	80.7 ± 9.3	91.1 ± 4.6	64.6 ± 13.1	n.d.	n.d.	25.9 ± 0.8	24.5 ± 9.5	55.1 ± 0.9

n.d.: not determined.

Table 4. Results of biochemical analysis of dry powder of ***S. maxima***.

contents of *S. platensis* increased to 70.90 ± 2.37% at sunrise, and became 57 ± 0.69% at sunset, indicating that it tends to remarkably decrease compared to the daytime [59]. On the contrary, it was reported that CHO content was higher at the sunset time (33.81 ± 0.66%) than the sunrise time (19.48 ± 1.48%). Thus, it would be necessary to study the maintenance of protein content by application of phosphate-feed condition and LED illumination environment after the early fall when temperature of culture medium and amount of sunlight decline.

Year-round contents of phycocyanin *S. maxima* produced in the present study were 12–93 mg/g. Phycocyanin content (mg/g) of *S. maxima* was 28.5 ± 0.9 mg/g in April 2011 when culture was started. Then, it gradually decreased and the mean content became 14.8 ± 3.1 mg/g in June, which was the lowest. It showed a trend of increase from July, and the mean phycocyanin concentration of September 2012 was 91.1 ± 4.6 mg/g, which was the maximum of the year. Thereafter, phycocyanin contents again decreased and recorded 24.5 ± 9.5 mg/g in its mean value in February 2012. Chlorophyll-*a* content of *S. maxima* was 6.1 ± 0.1 mg/g in April 2011 when culture was started, and then the mean content was 6.3 ± 2.0 mg/g in August when radiation amount was relatively high, which was the highest of the year. Afterwards, chlorophyll-*a* concentration decreased to 1.8 ± 0.6 mg/g in February 2012, which was the lowest of the year. However, although content of chlorophyll-*a* was at the highest in the summer similar to the correlation between year-round contents of phycocyanin and amount of sunlight, there was no consistent year-round significance. In general, cell growth and phycocyanin production are closely related with light conditions [60, 61]. However, phycocyanin content was the highest in September 2011 when light conditions were the best in the present study, though there was no consistent significance throughout the year. Chlorophyll-*a* contents showed 6.3 ± 2.0 mg/g in August 2011, the maximum value, and 1.7 ± 0.1 mg/g in January 2012, the minimum value. It has been reported that as the light energy that microalgae received increased, chlorophyll contents also significantly increased [62], and the present study also showed the highest chlorophyll-*a* content in August when radiation amount was relatively high. Despite the significance of monthly pigment contents, however, there was no constant year-round significance between pigments and amount of sunlight.

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4. Conclusion

A glass greenhouse pilot plant for microalgal culture fitting to temperate climate was designed based on 3D modeling designing BIM technology in KIOST. The bottom of the raceway system was placed 600 mm deep into the ground, and culture depth was kept at 400 mm, so that heat energy was efficiently stored in order to maintain thermal effects for a long time, and its structure was helpful in maintaining optimal temperature even in the winter. *S. maxima* was continuously cultured for a year in batch culture without further supply of nutrients, and the raceway system with a glass greenhouse structure in the present chapter achieved the maximum aerial productivity compared with most previous studies.

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Conflict of interest

The authors declare no conflicts of interest.

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Nomenclature

AOAC	the association of official analytical chemists
BIM	building information modeling
CFD	computational fluid dynamics
CHO	carbohydrate
GF/C	glass microfiber

KIOST	Korea Institute of Ocean Science and Technology
KWh	kilowatt hour
NIST	National Institute of Standards and Technology
ORS	open raceway system
PBR	photobioreactor
PE	polyethylene
PSU	practical salinity unit
RPM	revolutions per minute

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Chapter 5

Cyanophycin: A Nitrogen-Rich Reserve Polymer

Björn Watzer and Karl Forchhammer

Additional information is available at the end of the chapter

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Abstract

Cyanophycin is a nitrogen/carbon reserve polymer present in most cyanobacteria as well as in a few heterotrophic bacteria. It is a non-ribosomally synthesized polyamide consisting of aspartate and arginine (multi-L-arginyl-poly-L-aspartic acid). The following chapter provides an overview of the characteristics and occurrence of cyanophycin in cyanobacteria. Information about the enzymes involved in cyanophycin metabolism and the regulation of cyanophycin accumulation is also summarized. Herein, we focus on the main regulator, the P_{II} signal transduction protein and its regulation of arginine biosynthesis. Since cyanophycin could be used in various medical or industrial applications, it is of high biotechnological interest. In the last few years, many studies were published aiming at the large-scale production of cyanophycin in different heterotrophic bacteria, yeasts and plants. Recently, a cyanobacterial production strain has been reported, which shows the highest so ever reported cyanophycin yield. The potential and possibilities of biotechnological cyanophycin production will be reviewed in this chapter.

Keywords: cyanophycin, cyanophycin synthetase, cyanophycinase, nitrogen reserve, polyamide, L-arginine, L-aspartate, P_{II} protein

1. Introduction

Cyanophycin, abbreviated CGP (cyanophycin granule peptide), is next to poly-γ-glutamic acid and poly-ε-lysine, the third polyamino acid known to occur in nature [1]. It serves as a nitrogen/carbon reserve polymer in many cyanobacterial strains as well as in a few heterotrophic bacteria. CGP consists of the two amino acids, aspartate and arginine, forming a poly-L-aspartic acid backbone with arginine side chains. The arginine residues are linked to the β-carboxyl group of every aspartyl moiety via isopeptide bond [2].

CGP was discovered in 1887 by the botanist Antonio Borzi during microscopic studies of filamentous cyanobacteria [3]. He observed opaque and light scattering inclusions by using light microscopy and created the name *cianoficina*. Early electron microscopic studies showed a strong structure variation of the CGP granules, depending on the fixatives and poststains used during electron microscopic examinations [4, 5]. This led to a controversy about the ultrastructure of these inclusions until the 1970s. Later, electron microscopic studies described CGP granules as membrane less, electron dense and highly structured cytoplasmic inclusions [6, 7].

With a C/N ratio of 2:1, CGP is extremely rich in nitrogen and consequently an excellent nitrogen storage compound. During the degradation of CGP and subsequent degradation of arginine, a function as energy source was also proposed [8].

2. CGP occurrence

Most cyanobacteria, including unicellular and filamentous, as well as diazotrophic and non-diazotrophic groups are able to accumulate CGP (**Figure 1**).

In non-diazotrophic cyanobacteria, the amount of CGP is usually less than 1% of the cell dry mass during exponential growth. CGP accumulates conspicuously under unbalanced growth conditions including stationary phase, light stress or nutrient limitation (sulfate, phosphate or potassium starvation) that do not involve nitrogen starvation [9, 10]. Under such unbalanced conditions, the amount of CGP may increase up to 18% of the cell dry mass [10]. During the recovery from nitrogen starvation by the addition of a usable nitrogen source, CGP is transiently accumulated [11, 12].

In the unicellular diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142, nitrogen fixation and photosynthesis can coexist in the same cell, but temporarily separated. The nitrogen-fixing enzyme, nitrogenase, is highly sensitive to oxygen. Nitrogen fixation occurs in dark periods and the fixed nitrogen is stored in CGP. In the light period, when photosynthesis is performed, the CGP is degraded to mobilize the fixed nitrogen [13]. Transient CPG accumulation during dark periods was also reported in the filamentous cyanobacterium *Trichodesmium* sp., which has a high abundance in tropical and subtropical seas and is an important contributor to global N and C cycling [14].

Furthermore, in heterocysts of diazotrophic cyanobacteria of the order *Nostocales,* polar nodules consisting of CGP are deposited at the contact site to adjacent vegetative cells [15] (**Figure 1**). The heterocystous CGP seems to be involved in transport of fixed nitrogen to the adjacent photosynthetically active vegetative cell. CGP catabolic enzymes are present at significantly higher levels in vegetative cells than in heterocysts. Moreover, CGP could serve as a sink for fixed nitrogen in the heterocyst to avoid feedback inhibition from soluble products of nitrogen fixation [16, 17]. In *Anabaena* sp. PCC 7120 and *Anabaena variabilis,* mutational studies have shown that strains lacking CGP synthetic genes are little affected in diazotrophic growth under standard laboratory conditions [15, 18]. However, a growth defect was observed under high light conditions [15]. Moreover, diazotrophic growth is significantly decreased in strains that are unable to degrade CGP [16, 18].

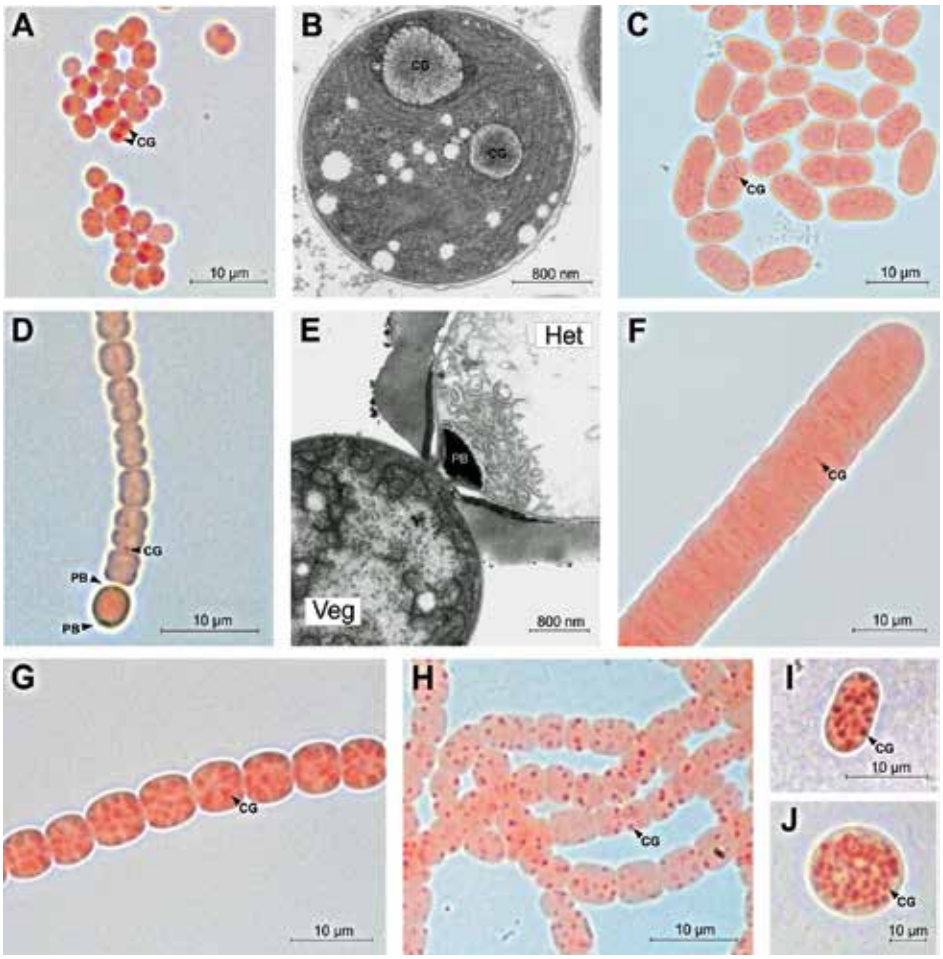

Figure 1. Light and electron microscopic pictures of CGP accumulating cyanobacteria. In light microscopic pictures, CGP was stained using the Sakaguchi reaction [10]. The intensity of the red color indicates the amount of arginine. Dark red to purple dots are CGP granules [CG]. (A) and (B) Phosphate starved *Synechocystis* sp. PCC 6803 in light and transmission electron microscopy, respectively. (C) *Cyanothece* sp. PCC 7424 cultivated in presence of nitrate and continuous light. (D) Filament of diazotrophic growing *Anabaena* sp. PCC 7120 with terminal heterocyst containing polar bodies [PB]. (E) Transmission electron micrographs of a heterocyst and adjacent vegetative cell from *Anabaena* sp. PCC 7120, showing a GCP consisting polar body [PB]. (F) *Oscillatoria* sp. cultivated with nitrate supplementation, showing small CGP granules. (G) Phosphate starved *Anabaena variabilis ATCC 29413* under nitrate supplemented growth. (H) *Nostoc punctiforme ATCC 29133* under phosphate starvation and nitrate supplementation. (I) and (J) Mature akinetes of *Anabaena variabilis ATCC 29413* and *Nostoc punctiforme ATCC 29133*, respectively.

Akinetes are resting spore-like cells of a subgroup of heterocyst-forming cyanobacteria for surviving long periods of unfavorable conditions. During akinete development, the cells transiently accumulate storage compounds, namely glycogen, lipid droplets and CGP [19, 20] (**Figure 1**). CGP granules also appear during germination of dormant akinetes [21]. *Anabaena variabilis* akinetes lacking CGP granules were also able to germinate. This behavior agrees with early observations that CGP is not the direct nitrogen source for protein biosynthesis and therefore not essential for akinete germination [21, 22].

CGP was formally thought to be unique in cyanobacteria. In 2002, Krehenbrink et al. and Ziegler et al. discovered through evaluation of obligate heterotrophic bacteria genomes that many heterotrophic bacteria possess CGP synthetase genes [23, 24]. Genes of CGP metabolism occur in a wide range of different phylogenetic taxa and not closely related to cyanobacteria [25].

3. CGP characteristics

In 1971, Robert Simon isolated CGP granules for the first time by using differential centrifugation. Along with this study, CGP has shown its special and unique solubility behavior [26]. CGP is insoluble at physiological ionic strength and at neutral pH, but soluble in solutions which are acidic, basic or highly ionic. In non-ionic detergents such as Triton X-100, CGP is insoluble; however, in ionic detergents like SDS, it is soluble [6]. Present-day CGP extraction methods are based on its solubility at low pH and insolubility at neutral pH [27].

The chemical structure of CGP was proposed in 1976 by Simon and Weathers [2]. According to this model, CGP has a polymer backbone consisting of α-linked aspartic acid residues. The α-amino group of arginine is linked via isopeptide bonds to the β-carboxylic group of every aspartyl moiety. Because every aspartate residue is linked to an arginine residue, CGP contains equimolar amounts of aspartate and arginine [2]. This structure has been confirmed via enzymatic degradation studies. CGP-degrading enzymes (see below) release β-Asp-Arg dipeptides [28]. CD spectroscopy data suggest that the acid-soluble and neutral insoluble forms of CGP have similar conformations. Both forms contain substantial fractions of β-pleated sheet structure [29].

Cyanobacterial CGP has a molecular weight and polydispersity ranging from 25 to 100 kDa [26]. In contrast, the native CGP producer *Acinetobacter* sp. ADP1 synthesizes CGP with a lower molecular weight ranging from 21 to 28 kDa [30]. Recombinant bacteria or genetically engineered yeast harboring heterologous expression of cyanobacterial CGP synthesis genes also show a lower molecular weight of 25–45 kDa [27, 31]. Transgenic plant-produced CGP also shows a reduced polydispersity between 20 and 35 kDa [32]. A possible explanation would be that cyanophycin synthesis in the native cyanobacterial background involves additional factors contributing the polymer length. These additional factors should also be absent in *Acinetobacter* sp. ADP1.

Native CGP is exclusively composed of aspartate and arginine. By contrast, in CGP isolated from recombinant *E. coli* expressing cyanophycin synthetase (see below) from *Synechocystis* sp. PCC 6803, besides aspartate and arginine, lysine has been found [33]. The amount of incorporated lysine in CGP influences its solubility behavior. Recombinant CGP with a high lysine amount (higher than 31 mol%) is soluble at neutral pH [34].

4. CGP metabolism

4.1. Cyanophycin synthetase

CGP is non-ribosomally synthesized from aspartate and arginine by cyanophycin synthetase (CphA1) (**Figure 2**). In 1976, CphA1 was enriched and characterized for the first time by

Simion [35]. The enzyme incorporates aspartate and arginine in an elongation reaction, which requires ATP, KCl, $MgCl_2$ and a sulfhydryl reagent (β-mercaptoethanol or DTT). For its activity, CphA1 needs a so far unknown CGP primer, as a starting point of the elongation reaction [35]. By using synthetically primers, Berg et al. could show that a single building block of CGP (β-Asp-Arg) does not serve as an efficient primer for CphA1 elongation reaction in vitro. The primers need to consist of at least three Asp-Arg building blocks $(\beta\text{-Asp-Arg})_3$ to detect CphA1 activity [36]. Other peptides, like cell wall or other cellular components, have been suggested to serve as an alternative priming substance for the CphA1 reaction [37]. This could be an explanation for the functionality of CGP synthesis in recombinant bacteria, without the ability to produce native CGP primers [38]. Interestingly, the CphA1 of *Thermosynechococcus elongatus* strain BP-1 shows primer-independent CGP synthesis [39].

Today, CphA1 enzymes from several bacteria, including cyanobacteria and heterotrophic bacteria, have been purified and characterized [33, 39–42]. The molecular mass of the characterized CphA1 enzymes ranges from 90 to 130 kDa. The active form of CphA1s from *Synechocystis* sp. PCC6308 and *Anabaena variabilis* PCC7937 is most likely homodimeric [33, 41], while the primer-independent CphA1 from *Thermosynechococcus elongatus* strain BP-1 forms a homotetramer [39]. The primary structure of cyanobacterial CphA1 can be divided into two regions [33]. The C-terminal region shows sequence similarities to peptide ligases that include murein ligases and folyl poly-γ-glutamate ligase. The N-terminal part of CphA1 shows sequence similarities with another superfamily of ATP-dependent ligases that include carboxylate-thiol

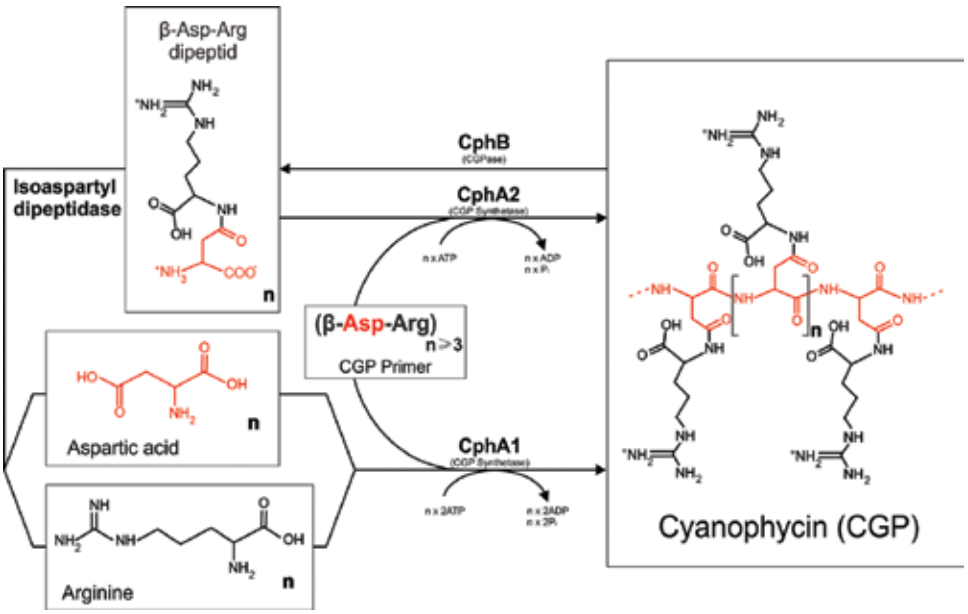

Figure 2. Schematic illustration of CGP metabolism in cyanobacteria. CGP is synthesized from aspartate and arginine by CGP synthetase (CphA1) in an ATP-depending elongation reaction using CGP primers, containing of at least three Asp-Arg building blocks. Intracellular CGP degradation is catalyzed by the CGPase (CphB). The β-Asp-Arg dipeptides resulting from cleavage of CGP are further hydrolyzed by isoaspartyl dipeptidase, releasing aspartate and arginine. In many nitrogen-fixing cyanobacteria, an additional CGP synthetase is present, termed CphA2. CphA2 can use β-aspartyl-arginine dipeptides to resynthesize CGP.

and carboxylate-amine ligase. Since the C- and N-terminal parts show similarity to different superfamilies of ATP-dependent ligases, two ATP-binding sites and two different active sites have been predicted [36]. In vitro experiments revealed that arginine is probably bound in the C-terminal and aspartate in the N-terminal active site [43].

The mechanism of CGP synthesis by CphA1 has been suggested by Berg et al. in 2000, by measuring the step-wise incorporation of amino acids to the C-terminus of the CGP primer. The putative CGP elongation cycle starts at the C-terminal end of the poly-aspartate backbone. First, the carboxylic acid group of the poly-aspartate backbone is activated by transfer of the γ-phosphoryl group of ATP. In the second step, one aspartate is bound at the C-terminus of the growing polymer by its amino group, forming a peptide bound. Subsequently, the intermediate $(\beta\text{-Asp-Arg})_n$-Asp is transferred to the second active site of CphA1 and phosphorylated at the β-carboxyl group of the aspartate. Finally, the α-group of arginine is linked to the β-carboxyl group of aspartate, forming an isopeptide bound [36].

Various CphA1 enzymes have been characterized with respect to their substrate affinity and specificity. For CphA1 of *Synechocystis* sp. PCC 6308, apparent K_m values were determined to be 450 μM for aspartate, 49 μM for arginine, 200 μM for ATP and 35 μg/ml CGP as priming substance. The lower K_m of arginine compared to aspartate indicates a higher affinity of CphA1 towards arginine. During the in vitro reaction, CphA1 converts per mol incorporated amino acid 1.3 ± 0.1 mol ATP to ADP. The optimal reaction conditions of this enzyme were at pH 8.2 and 50°C [41].

CphA homologs are widely distributed in eubacteria. In silico analysis proposes 10 different groups of cyanophycin synthetases [25]. In cyanobacteria, cyanophycin synthetases of group I–III (CphA, CphA2 and CphA2′) can be found.

Recently, the function of a cyanophycin synthetase of group II (CphA2) has been characterized. Most non-diazotrophic cyanobacteria use a single type of cyanophycin synthetase (CphA1). However, in many nitrogen-fixing cyanobacteria, an additional version of CphA is present, termed CphA2. In 2016, Klemke et al. resolved the function of CphA2 [44]. Compared to CphA1, CphA2 has a reduced size and just one ATP-binding site. CphA2 uses the product of CGP hydrolysis, β-aspartyl-arginine dipeptide as substrate to resynthesize cyanophycin, consuming one molecule of ATP per elongation. A mutant lacking CphA2 shows only a minor decrease in the overall CGP content. However, a CphA2-deficient mutant displays similar defects under diazotrophic and high light conditions than a CphA1 mutant [15, 44]. This observation suggests that the apparent “futile cycle” of CGP hydrolysis and immediate repolymerization is probably of physiological significance in the context of nitrogen fixation [17].

4.2. Cyanophycinase

Since 1976, it is known that CGP is resistant against hydrolytic cleavage by several proteases or arginase [2, 45]. This resistance is probably due to the branched structure of CGP [38]. Therefore, the presence of a highly specified peptidase for CGP hydrolysis was suggested.

In 1999, Richter et al. reported a CGP hydrolyzing enzyme from the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, called CphB [28] (**Figure 2**). During this study, CphB was purified

and studied in detail. CphB is a 29.4 kDa C-terminal exopeptidase, catalyzing the hydrolyzation of CGP to β-Asp-Arg dipeptides [28]. Based on sequence analysis and inhibitor sensitivity to serine protease inhibitors, CphB appears to be a serine-type exopeptidase related to dipeptidase E (PepE) [28]. According to its sequence, CphB contains a serine residue within a lipase box motive (Gly-Xaa-Ser-Xaa-Gly). The serine residue together with a glutamic acid residue and a histidine residue forms the catalytic triad, which is typical for serine-type peptidases [28]. In 2009, the crystal structure has been solved at a resolution of 1.5 Å, showing that CphB forms a dimer. Site-directed mutagenesis confirms that CphB is a serine-type peptidase, consisting of a conserved pocket with the catalytic Ser at position 132 [46]. Structure modeling indicates that the cleavage specificity occurs due to an extended conformation in the active site pocket. The unique conformation of the active site pocket requires β-linked aspartyl peptides for binding and catalysis, preventing CphB from non-specific cleavage of other polypeptides next to CGP [46].

In addition to CphB, which catalyzes the intracellular cleavage of CGP, other versions of cyanophycinase exist, catalyzing the extracellular hydrolysis of CGP. In 2002, Obst et al. isolated several Gram-negative bacteria from different habitats, which were able to utilize CGP as a source of carbon and energy [47, 48]. One isolate was affiliated as *Pseudomonas anguilliseptica* strain BI. In the supernatant of a *Pseudomonas anguilliseptica* culture, a cyanophycinase was found and purified, called CphE [47]. CphE exhibits a high specificity for CGP; however, proteins were not or only marginally hydrolyzed. Degradation products of CphE are β-Asp-Arg dipeptides. Inhibitor sensitivity studies indicated that the catalytic mechanism of CphE is related to serine-type proteases. CphE from *Pseudomonas anguilliseptica* strain BI exhibits an amino acid sequence identity 27–28% to intracellular CphB enzymes of cyanobacteria [47]. Today, extracellular CGPases has been found in a high variety of bacteria including Gram-positive, Gram-negative, aerobic and anaerobic strains. This indicates that the extracellular cleavage and utilization of CGP as carbon, nitrogen and energy source is a common principle in nature [47–53].

In 2007, in silico analysis showed that CphB homologs are widely distributed in eubacteria, proposing eight different groups including intracellular and extracellular CGPases. CGPases from cyanobacteria belong to group I, II and partially group III ($CphB_{1–3}$). Groups IV–VIII, including CphE, are present in a large variety of non-photosynthetic bacteria [25].

4.3. Aspartyl-arginine dipeptidase

The last step in catabolism of CGP is the cleavage of β-Asp-Arg dipeptides to monomeric amino acids, arginine and aspartate (**Figure 2**). In 1999, Richter et al. found β-Asp-Arg dipeptides hydrolyzing activity in extracts of *Synechocystis* sp. PCC 6803 [28]. In *Synechocystis* sp. PCC 6803, the ORF sll0422 as well as ORF all3922 from *Anabaena* sp. PCC 7120 is annotated as "plant-type asparaginase," because of sequence similarities to the first cloned asparaginase from plants [54]. During characterization of plant-type asparaginase in general, including Sll0422 and All3922, Hejazi et al. were able to show that these enzymes are able to hydrolyze a wide range of isoaspartyl dipeptides [55]. Isoaspartyl peptides arise from two biological pathways: First, proteolytic degradation of modified proteins containing isoaspartyl residues and second, as primary degradation product of CGP cleavage from CGPases. Thus, the plant-type

asparaginases, Sll0422 and All3922, have not only a function in asparagine catabolism but also in the final step of CGP and protein degradation [55].

The mature isoaspartyl dipeptidases of *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 consist of two protein subunits that are generated by autocleavage of the primary translation product between Gly-172 and Thr-173 (numbering according to *Synechocystis* sp. PCC 6803) within the conserved consensus sequence GT(I/V)G [55]. The native molecular weight of approximately 70kD of this enzyme suggests that it has a subunit structure of $\alpha_2\beta_2$ (α derived from the N-terminal part and β from the C-terminal part of the precursor) [55].

In *Anabaena* sp. PCC 7120, all genes involved in CGP metabolism as well as the isoaspartyl dipeptidases All3922 are expressed in vegetative cells and heterocysts but in different expression levels. Both, CGP synthetases and CGPases are much higher expressed in heterocysts than in vegetative cells [56]. However, asparaginase All3922 is present in significantly lower levels in heterocysts than in vegetative cells [57]. A deletion of All3922 in *Anabaena* sp. PCC 7120 causes an increased accumulation of CGP and β-Asp-Arg dipeptides. Furthermore, a deletion mutant shows an impaired diazotrophic growth similar to the phenotype known from CphB deletion mutants in *Anabaena* sp. PCC 7120 [18, 57]. This observation implies that the first step of CGP catabolism, the cleavage catalyzed by CphB, takes place in the heterocyst. The released β-Asp-Arg dipeptides are transported to the adjacent vegetative cells. Isoaspartyl dipeptidase All3922, present in the vegetative cells, cleaves the β-Asp-Arg dipeptides and releases monomeric aspartate and arginine [57]. When CGP synthesis is not possible, due to a deletion of CphA, arginine and aspartate might be transferred directly from heterocysts. This explains the minor effects on diazotrophic growth in a CphA deletion mutant [15]. These results identified β-Asp-Arg dipeptides as nitrogen vehicle in diazotrophic heterocyst forming cyanobacteria, next to glutamine and arginine alone or with aspartate [57–59]. A benefit of β-Asp-Arg dipeptides as nitrogen transport substance is avoiding the release of free arginine and aspartate in the heterocyst. This indicates that CGP metabolism has evolved in multicellular heterocyst-forming cyanobacteria to increase the efficiency of nitrogen fixation [57].

5. CGP regulation

5.1. Genetic organization of CphA and CphB

Usually, genes involved in CGP metabolism are clustered. The organization of these clusters can be different, depending on the respective organism [25]. In *Synechocystis* sp. PCC 6803, *cphA* and *cphB* are adjacent; however, they are expressed independently [60]. A hypothetical protein named slr2003 is located downstream of *cphA* and is transcribed in a polycistronic unit with *cphA* [60]. However, the function of Slr2003 is unknown. In the gene of CphB (slr2001), a small antisense RNA was detected (transcriptional unit 1486) [60].

In *Anabaena* sp. PCC 7120, two clusters containing CphA and CphB were identified [18]. In the *cph1* cluster, *cphB1* and *cphA1* were expressed under ammonia and nitrate supplemented growth, but the expression of both genes was higher in the absence of combined nitrogen in

heterocysts and vegetative cells. In the *cph1* operon, *cphB1* and *cphA1* were cotranscribed. In addition, *cphA1* can be expressed from independent promoters, of which one is constitutive and the other regulated by the global nitrogen control transcriptional factor NtcA [18].

In cluster *cph2*, the *cphB2* and *cphA2* genes were found in opposite orientation and both genes were expressed monocistronically. The genes were expressed under conditions of ammonia, nitrate or N_2 supplementation, but the expression was higher in the absence of ammonia. Generally, the expression of the *cph2* is lower compared to *cph1* [18].

In addition to these two gene clusters, a third set of ORFs containing putative *cphA* and *cphB* genes was found in *Nostoc punctiforme* PCC 73102 and *Anabaena variabilis* ATCC 29413 [25].

5.2. Dependence of CGP metabolism on arginine biosynthesis

Generally, CGP accumulation is triggered by cell growth arresting stress conditions, such as entry into stationary phase, light or temperature stress, limitation of macronutrients (with the exception of nitrogen starvation) or inhibition of translation by adding antibiotics like chloramphenicol [9, 10, 61]. All of these CGP triggering conditions result in a reduced or arrested growth. In exponential growth phase the amino acids arginine and aspartate are mostly used for protein biosynthesis with the consequence of a low intracellular level of free amino acids. Under growth-limiting conditions, protein biosynthesis is slowed down, which yields an excess of monomeric amino acids in the cytoplasm, triggering the CGP biosynthesis [10].

CGP accumulation also requires an excess of nitrogen. For the filamentous cyanobacterium *Calothrix* sp. strain PCC 7601, it was shown that CGP accumulation occurs preferably in the presence of ammonia [62]. The addition of amino acids to the media further increased CGP formation [63]. During process optimization studies for heterotrophic CGP production in the strain *Acinetobacter calcoaceticus* ADP1, it was shown that addition of arginine to the medium as sole carbon source increased CGP accumulation drastically. When, in *A. calcoaceticus* strain ADP1, CGP synthesis is induced by phosphate starvation, it accounts to 3.5% (w/w) of the cell dry matter (CDM) with ammonia as nitrogen source. Additional supply of the medium with arginine increases the CGP amount to 41.4% (w/w) (CDM). Notably, a combined supply of arginine and aspartate has a much lower stimulating effect to CGP accumulation than arginine alone [30].

A potential link between regulation of arginine biosynthesis and GCP metabolism was suggested in many previous studies. In a transposon mutagenesis study in the filamentous cyanobacterium *Nostoc ellipsosporum*, an arginine biosynthesis gene, *argL*, was interrupted by a transposon. This mutation partially impairs arginine biosynthesis but does not strictly result in L-arginine auxotrophy. Without arginine supplementation, heterocysts failed to fix nitrogen, akinetes were unable to germinate and CGP granules did not appear. However, when both nitrate and arginine are present in the media, the impaired arginine biosynthesis is bypassed. Under this condition, the mutant could form CGP and was able to differentiate functional akinetes, which contained CGP granules [64].

In metabolic engineering studies of the CGP production strain *Acinetobacter calcoaceticus* ADP1, several genes related to the arginine biosyntheses or its regulation were modified to yield higher amounts of arginine. As a consequence, significant higher CGP production was observed [65].

Bacteria produce arginine from glutamate in eight steps. The first five steps involving N-acetylated intermediates lead to ornithine. The conversion of ornithine to arginine requires three additional steps [66]. The second enzyme of ornithine biosynthesis is the N-acetylglutamate kinase (NAGK), which catalyzes the phosphorylation of N-acetyl glutamate to N-acetylglutamylphosphate. NAGK catalyzes the controlling step in arginine biosynthesis [67]. NAGK activity is subjected to allosteric feedback inhibition by arginine and is, moreover, positively controlled by the P_{II} signal transduction protein (see below) [67, 68]. Maheswaran et al. showed that arginine production and the following CGP accumulation depend on the catalytic activation of NAGK by the signal transduction protein P_{II} [69]. In a P_{II}-deficient mutant of *Synechocystis* sp. PCC 6803, NAGK remained in a low activity state, which caused impaired CGP accumulation [69].

The nitrogen-regulated response regulator NrrA also has influence on arginine and CGP biosynthesis. An NrrA-deficient mutant in *Synechocystis* sp. PCC 6803 shows reduced intracellular arginine levels and, consequently, reduced CGP amount [70].

All these results and observations point towards arginine as main bottleneck of CGP biosynthesis, while aspartate plays a minor role. CGP accumulation occurs as a result of arginine enrichment in the cytoplasm. Reasons for increased arginine content in the cell are lowered protein biosynthesis as a result of various growth limiting conditions. Furthermore, an excess of nitrogen and energy sensed by P_{II} leads to NAGK activation and thereby increased arginine biosynthesis.

5.3. P_{II} regulation of arginine metabolism

The P_{II} signal transduction proteins are widely distributed in prokaryotes and chloroplasts, where they play a coordinating role in the regulation of nitrogen assimilatory processes [71–73]. For this purpose, P_{II} senses the energy status of the cell by binding ATP or ADP in a competitive way [74]. Binding of ATP and synergistic binding of 2-oxoglutarate (2-OG) allows P_{II} to sense the current carbon/nitrogen status of the cell [75]. 2-OG is the carbon skeleton for the GS/GOGAT reactions and thereby links the carbon and nitrogen metabolism in all domains of life [76, 77]. The pool size of 2-OG reacts quickly to changes in nitrogen availability, wherefore 2-OG is an indicator of the carbon/nitrogen balance [78, 79]. Depending on the nitrogen supply, P_{II} may be phosphorylated at the apex of the T-loop at position Ser49 [80, 81]. Binding of the effector molecules ATP, ADP and 2-OG as well as phosphorylation leads to conformational rearrangements of the large surface-exposed T-loop, P_{II}'s major protein-interaction structure [82]. These conformational states direct the interaction of P_{II} with its various interaction partners and thereby regulate the cellular C/N balance [83].

In cyanobacteria, P_{II} regulates the global nitrogen control transcriptional factor NtcA, through binding to the NtcA co-activator PipX [84]. In common with other bacteria, cyanobacterial P_{II} proteins can interact with the biotin carboxyl carrier protein (BCCP) of acetyl-CoA carboxylase (ACC) and thereby control the acetyl-CoA levels [85]. Furthermore, P_{II} controls arginine biosynthesis via regulation of NAGK [68, 69, 86].

P_{II} proteins form a cylindrical-shaped homotrimer with 12–13 kDa per subunits. The T-loop, a large and surface-exposed loop, protrudes from each subunit. The effector binding sites are positioned in the three inter-subunit clefts [87, 88]. If sufficient energy and nitrogen are available,

indicated by a high ATP and low 2-OG level, non-phosphorylated P_{II} forms an activating complex with NAGK.

The crystal structure of the P_{II}-NAGK complex from *Synechococcus elongatus* strain PCC 7942 revealed two P_{II} trimers sandwiching a NAGK homohexamer (trimer of dimers) [88]. Each P_{II} subunit contacts one NAGK subunit [88]. Two parts of P_{II} are involved in interaction with NAGK. The first structure, called B-loop, is located on the P_{II} body and interacts with the C-domain of NAGK subunit, involving residue Glu85. The interaction of the B-loop is the first step in complex formation. Second, the T-loop must adopt a bent conformation and insert into the interdomain cleft of NAGK [89]. This enhances the catalytic efficiency of NAGK, with the V_{max} increasing fourfold and the K_m for N-acetylglutamate decreasing by a factor of 10 [86]. Furthermore, feedback inhibition of NAGK by arginine is strongly decreased in the presence of P_{II} [86].

During P_{II} mutagenesis, a P_{II} variant was identified that binds constitutively NAGK in vitro. This P_{II} variant exhibits a single amino acid replacement, Ile86 to Asn86, hereafter referred as P_{II}(I86N) [89]. The crystal structure of P_{II}(I86N) has been solved, showing an almost identical backbone than wild-type P_{II}. However, the T-loop adopts a compact conformation, which is a structural mimic of P_{II} in the NAGK complex [89, 90]. Addition of 2-OG in the presence of ATP normally leads to a dissociation of the P_{II}-NAGK complex, however P_{II}(I86N) no longer responds to 2-OG [90].

The P_{II}(I86N) variant enables a novel approach of metabolic pathway engineering by using custom-tailored P_{II} signaling proteins. By replacing the wild-type P_{II} with a P_{II} carrying the mutation for I86N in *Synechocystis* sp. PCC 6803, it was possible to engineer the first cyanobacterial CGP overproducer strain. Strain BW86, containing the P_{II}(I86N) version, shows an increase of NAGK activity, which causes a more than 10-fold higher arginine content than the wild-type [10]. Under balanced growth conditions with nitrate as nitrogen source, strain BW86 accumulates up to 15.6 ± 5.4% CGP relative to the CDM, i.e., on average almost sixfold more than the wild type. Appropriate starvation conditions can further increase the CGP content of strain BW86 up to 47.4 ± 2.3% per CDM under phosphate starvation and 57.3 ± 11.1% per CDM under potassium starvation, without addition of arginine to the medium [10]. Furthermore, the CGP, which is produced by strain BW86, shows a high polydispersity ranging from 25 to 100 kDa, similar to the polydispersity of cyanobacterial wild-type CGP, which contrasts CGP from recombinant producer strains using heterologous expression systems with heterotrophic bacteria, yeasts or plants [10]. CGP isolated from those strains have a size ranging of 25–45 kDa [27, 31, 32].

6. Industrial applications

Industrial applications for CGP have previously mainly focused on chemical derivatives. CGP can be converted via hydrolytic β-cleavage to poly(α-L-aspartic acid) (PAA) and free arginine. PAA is biodegradable and has a high number of negatively charged carboxylic groups, making PAA to a possible substituent for polyacrylates [48, 50, 91]. PAA can be employed as antiscalant or dispersing ingredient in many fields of applications, including washing detergents or suntan lotions. Furthermore, PAA has potential application areas as an additive in paper, paint, building or oil industry [48, 50].

CGP can also serve as a source for dipeptides and amino acids in food, feed and pharmaceutical industry. The amino acids arginine (semi-essential), aspartate (non-essential) and lysine (essential) derived from CGP have a broad spectrum of nutritional or therapeutic applications. Large-scale production of these amino acids, as mixtures or dipeptides, is established in industry, with various commercial products already available on the market (reviewed by Sallam and Steinbuchel [92]).

Potential applications of non-modified CGP have been discussed but remain so far largely unexplored. This can partially be explained by the lack of research being conducted on the material properties of CGP. Recently in 2017, the first study regarding CGP material properties has been published. In this study, Khlystov et al. focused on the structural, thermal, mechanical and solution properties of CGP produced by recombinant *E. coli*, giving new insights in the nature of this polymer as bulk chemical [91]. They describe CGP as an amorphous, glassy polyzwitterion with high thermostability. The dry material is stiff and brittle. According to these properties, CGP could be used to synthesize zwitterionomeric copolymers or as reinforcing fillers [91].

7. Biotechnological production

Previous ventures to produce CGP in high amounts were mainly focused on heterotrophic bacteria, yeasts and plants as production host. These recombinant production hosts heterologously express CGP synthetase genes, mostly from cyanobacteria. In this way, heterotrophic bacteria, which are established in biotechnological industry including *E. coli*, *Corynebacterium glutamicum*, *Cupriavidus necator* (formally known as *Ralstonia eutropha*) and *Pseudomonas putida*, were used for heterologous production of CGP [93].

Strain *E. coli* DH1, containing *cphA* from *Synechocystis* sp. PCC6803, was used for large-scale production of CGP in a culture volume of up to 500 liter, allowing the isolation of CGP in a kilogram scale. During process optimization, the highest observed CGP content was 24% (w/w) per CDM. However, the synthesis of CGP was strongly dependent on the presence of complex components in the medium (terrific broth complex medium). In mineral salt medium, CGP accumulation only occurs in the presence of casamino acids [27]. An engineered version of CphA from *Nostoc ellipsosporum,* transformed in *E. coli*, shows a further increase in CGP production, up to 34.5% (w/w) of CDM. However, this production strain also requires expensive complex growth media to yield such a high amount of CGP [94].

Cupriavidus necator and *Pseudomonas putida* are known as model organisms for the industrial scale production of polyhydroxyalkanoates (PHA). Therefore, they have been considered as candidates for large scale CGP production [93, 95]. Metabolic engineering and process optimization studies of *Cupriavidus necator* and *Pseudomonas putida* harboring *cphA* from *Synechocystis* sp. PCC 6803 or *Anabaena* sp. PCC 7120 were performed. In these organisms, the accumulation of CGP is mainly depending on the origin of the *cphA* gene, the accumulation of other storage compounds like PHA as well as the addition of precursor components like arginine to the medium [96]. PHA-deficient mutants of *Cupriavidus necator* and *Pseudomonas putida* accumulate in general more CGP compared to the PHA containing strains [96]. During genetic modification of *cphA* expression in *Cupriavidus necator*, CGP accumulation turned out to be

strongly affected by the expression system. A stabilized multi-copy *cphA* expression system, using the KDPG-aldolase gene (*eda*)-dependent addiction system, allows cultivation without antibiotic selection. The multi-copy *cphA* expression results in a CGP yield between 26.9% and 40.0% (w/w) of CDM. The maximum amount of 40.0% (w/w) of CDM was observed in a 30- and 500-l pilot plant. In the absence of the amino acids arginine and aspartic acid in the medium, the CGP amount was still between 26.9% and 27.7% (w/w) of CDM [97].

The industrially established host *Saccharomyces cerevisiae* has also been used for CGP production, by expression of *cphA* from *Synechocystis* sp. PCC 6803. *S. cerevisiae* harboring *cphA* accumulated up to 6.9% (w/w) of CDM. Two CGP species were observed in this strain: water-soluble and the typical water-insoluble CGP. Furthermore, the isolated polymer from this transgenic yeast contained 2 mol% lysine, which can be increased up to 10 mol% when cultivation occurs with lysine in the medium [31]. During metabolic engineering studies, several arginine biosynthesis mutants have been analyzed concerning their CGP accumulation abilities. Surprisingly, strains with defects in arginine degradation accumulated only 4% CGP (w/w) of CDM; however, arginine auxotrophic strains were able to accumulate up to 15.3%. Depending on the cultivation conditions, between 30 and 90% of the extracted CGP was soluble at neutral pH. In addition to arginine, aspartate and lysine, further amino acids, such as citrulline and ornithine, have been detected in isolated CGP from different arginine biosynthesis mutants [98]. Furthermore, it was also possible to produce CGP and CGP derivates in *Pseudomonas putida* and the yeast *Pichia pastoris* [99, 100].

CGP and CGP derivates are important sources for β-dipeptides for several applications. A large-scale method was developed to convert CGP into its constituting β-dipeptides by using CphE from *Pseudomonas alcaligenes*. This allows the large-scale production of customized β-dipeptides, depending on the composition of the CGP derivates [92, 101].

Production of CGP has also been attempted in several transgenic plants. Here, ectopic expression of the primer-independent CphA from *Thermosynechococcus elongatus* BP-1 leads to an accumulation of CGP up to 6.8% (w/w) in tobacco leafs and to 7.5% (w/w) of CDM in potato tubers [102, 103]. CGP production and extraction in plants can be coupled with the production of other plant products like starch [103]. The peculiarities and challenges of plant-produced CGP have been reviewed by Nausch et al. [32].

Compared to bacteria that are used so far in biotechnological industry, cyanobacteria are unique as they use sunlight and CO_2 as energy and carbon source. Cyanobacteria have been identified as rich source of various biologically active compounds, biofertilizers, bioplastics, energy, food and feed [104]. Obviously, the importance of environmentally friendly production processes increases more and more. Hence, Cyanobacteria are expected to play a major role in future industry. *Synechocystis* sp. PCC 6803 strain BW86 is the first reported bulk chemical producing cyanobacterial strain in the literature. CGP production in *Synechocystis* BW86 does not require organic carbon or CGP precursor substances. Growth limiting conditions like phosphate and potassium starvation can further increase the CGP production up to 47.4 ± 2.3% and 57.3 ± 11.1% per CDM, respectively. The studies of Trautmann et al. showed that strain BW86 can be cultivated in flat plate photobioreactors (Midiplate reactor system [105]). During this optimization study, the optimal light intensity as well as the phosphate concentration was determined to maximize CGP synthesis. Under optimal production conditions, highest amount of CGP was around 40% of CDM with a total yield of 340 mg CGP per liter in 9 days [106].

The main bottleneck of CGP production in Cyanobacteria is the relatively slow growth rate, which is much lower than in biotechnologically established bacteria. Conventional cultivation methods of cyanobacteria reach a biomass of roughly 1 g dry mass per liter [107]. To overcome this limitation, a new cultivation method was developed, using a two-tier vessel with membrane-mediated CO_2 supply. By using this cultivation setup, it was possible to enable rapid growth of *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 up to 30 g CDM per liter [108]. *Synechocystis* sp. PCC 6803 strain BW86 was also used in this high-density cultivation setup. During this study, CGP amounts up to 1 g per liter were reached in 96 h. This is approximately four times higher compared to the maximum CGP yield observed during conventional cultivation after 12 days [106, 109].

In comparison, the recombinant *E. coli* strain DH1 harboring *cphA* from *Synechocystis* sp. PCC 6803 produces between 6.7 and 8.3 g CDM per liter culture in 16 h. CGP amounts during this fed-batch fermentations were between 21 and 24% of the CDM [27], resulting in a CGP production rate of 87.9 to 124.5 mg/l and hour. Although this exceeds the production rate in *Synechocystis* sp. PCC 6803 strain BW86 by a factor of 10, the recombinant *E. coli* requires terrific broth complex medium, while *Synechocystis* sp. PCC 6803 strain BW86 is cultivated in simple mineral medium and additionally sequesters hazardous greenhouse gas CO_2. Considering these super ordinate factors, production of biopolymers with cyanobacteria may in fact become an alternative to heterotrophic bacteria.

8. Conclusions

CGP is well researched and its occurrence in cyanobacteria is known for more than 100 years. However, many questions are still open. Most obviously, the cell biology of the CGP granules remains largely unknown. In the last decades, research on CGP mainly focused on biotechnological purposes, like strain or process optimization. Most work has been carried out with short-chain CGP from recombinant producer strains; however the biophysical properties of the long-chain native CGP remain largely unexplored. So far, heterotrophic bacteria were mainly used to produce industrial biocompounds including CGP. In this chapter, we discussed the possibility of a cyanobacterial CGP production strain. The main disadvantages of cyanobacteria, their slower growth and the low abundance of product can be compensated using genetic engineering together with appropriate production processes. Future industry has to cope with the manifold challenges to counteract environmental pollution and climate change. The use of cyanobacteria in CGP production and, more generally, in biotechnological applications for bioproduct synthesis provides an environmentally friendly alternative to conventional biotechnological approaches.

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Conflict of interest

The authors declare that they have no competing interests.

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Chapter 6

The Challenge of Iron Stress in Cyanobacteria

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Additional information is available at the end of the chapter

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Abstract

Iron is an essential nutrient for most living organisms. Due to the low solubility of ferric iron at physiological pH, the transition from an anaerobic atmosphere to the actual oxidant environment caused a dramatical decrease of iron bioavailability. Therefore, most organisms had to adapt their lifestyle to survive under an iron-depleted environment. In cyanobacteria, the electron transport chains involved in photosynthesis and respiration, as well as the enzymes involved in nitrogen metabolism have a high content of iron. Hence, cyanobacterial iron requirements are much higher than those of heterotrophic organisms. In this chapter, we revise different strategies developed by this important group of microorganisms to cope with iron deficiency, as well as the regulatory networks involved in the homeostasis of this indispensable element.

Keywords: cyanobacteria, iron stress, regulation, photosynthesis, nitrogen metabolism, cross-talk, cyanotoxin production

1. Introduction

The biological importance of iron almost entirely resides in its incorporation into proteins, either as a mono- or binuclear species, or as part of iron-sulfur clusters and heme groups. Through these forms, iron acts as a cofactor of a plethora of crucial enzymes and electron carriers involved in major biological processes including photosynthesis, respiration, tricarboxylic acid cycle, DNA biosynthesis and nitrogen fixation, among others [1]. Despite iron is the fourth most abundant element on earth crust, its bioavailability is extremely limited because of its poor solubility in the actual oxygenic atmosphere. Hence, whereas free Fe^{3+}

concentration ranges from 10^{-9} to 10^{-18} M, virtually all living microorganisms require a minimum effective concentration of 10^{-8} M to live and growth, and at least 10^{-7} to 10^{-5} M to achieve optimal growth [1].

Iron limitation is a challenge of particular importance in cyanobacteria, being one of the main limiting factors of ocean primary productivity [2]. Cyanobacteria have an absolute dependence of iron for growth and optimal development of their major physiological processes, particularly photosynthesis and nitrogen fixation. Iron serves as a cofactor for every membrane-bound protein complex and other mobile electron carriers within the photosynthetic apparatus [3], which determines an iron quota about 10 times higher than that exhibited by a similarly sized non-photosynthetic bacterium [4]. Additionally, diazotrophic cyanobacteria have significant further iron requirements compared with other phototrophs due to the abundance of iron-containing enzymes in the nitrogen-fixation machinery [5]. Although iron plays a key role in cyanobacterial physiology, an excess of free intracellular iron is extremely deleterious because it catalyzes the formation of reactive oxygen species (ROS) through Fenton reactions, leading to oxidative stress [6]. Likewise, iron starvation leads to significant increase in ROS and induces oxidative stress in cyanobacteria [7]. Hence, iron uptake and metabolism must be tightly regulated in order to ensure suitable supply maintaining the intracellular concentration within nontoxic levels [8, 9].

To cope with the usually frequent periods of iron starvation in nature, cyanobacteria have evolve efficient strategies which imply changes in the transcription of a plethora of genes, resulting among other changes in a deep rearrangement of the photosynthetic machinery [10] and the induction of the mechanisms involved in iron uptake. Thus, the transcription of genes coding for several TonB-dependent outer membrane transporters, periplasmic ferric-binding proteins, ATP-binding permeases as well as enzymes involved in siderophore biosynthesis will depend on iron availability [9, 11, 12].

Since an effective balance between iron acquisition and protection against oxidative stress is crucial for cell survival, as occurs in most Gram-negative and several Gram-positive bacteria, in cyanobacteria iron homeostasis is controlled by a global transcriptional regulator known as Fur, which stands for ferric uptake regulator [9, 13, 14]. Fur typically acts as a transcriptional repressor, which senses intracellular free iron and modulates transcription in response to iron availability [1]. Fur not only controls the expression of iron acquisition and storage systems, but also a wide set of genes and operons belonging to a broad range of functional categories, thereby contributing to couple iron availability to major physiological processes in cyanobacteria [14–17]. In this chapter, we revise the strategies of these photosynthetic bacteria to face the challenge of iron starvation. We put special emphasis in the transcriptional and physiological changes triggered by iron starvation in this group of microorganisms. Details on cyanobacterial iron metabolism and control of iron homeostasis as well as their connections with other cellular processes are discussed.

2. Classical strategies to overcome iron starvation situations

Cyanobacteria evolved very efficient mechanisms to cope with iron deficiency. Iron deprivation triggers a variety of responses that range from upregulation of the iron acquisition systems to

reduction or substitution of structures or molecules. At the physiological level, Strauss [18] categorized the responses as retrenchment (reduction of cell size, loss of phycobilisomes, ultrastructural changes and pigment changes), compensation (as the synthesis of flavodoxin, playing ferredoxin role, expression of *isiA* gene) and acquisition (induction of iron acquisition systems). Accommodation to iron deficiency requires changes in the expression of a large number of genes of many metabolic pathways, some of them not obviously related with iron metabolism, such as respiration, photosynthesis, nitrogen metabolism, glycolysis, tricarboxylic acids cycle, amino acid synthesis, synthesis of toxins and antioxidant defenses. Those changes highlight the responses associated to iron deficiency [9, 19]. It is important to consider that the responses are going to be different depending on the stress threshold: moderate, severe or extreme.

2.1. Rearrangement of photosynthetic electron transport chain under iron starvation conditions

Many photosynthetic components are iron-containing proteins, and also iron is involved in chlorophyll synthesis. Chlorophyll level is affected by iron availability, so the photosynthetic machinery may be diminished or even dismantled if the deficiency occurs suddenly, as in laboratory experiments. In general, populations living in limiting environments adapt its chlorophyll synthesis to the bioavailability, and the chlorophyll per cell is lower. Iron deficiency adaptation implies a reduction of the linear photosynthetic electron transport and enhances respiratory electron transport [20, 21] as well as a concomitant increase of the cyclic photophosphorylation [22]. Moreover, under iron deficiency, several responses to oxidative stress have been described, evidencing the link between iron starvation and oxidative stress, with photosystems specially affected [7, 23]. Consistently, several photosynthetic and oxidative defense genes have been identified as regulated by iron availability [9, 14, 24]. Among the iron-induced genes, *isiAB* [13] and *idiAB* products are playing key roles in the adaptability of the photosynthetic machinery to optimize its function at low iron availability.

2.1.1. IsiA and IsiB proteins

In *Synechococcus* sp., the *isiAB* operon is transcriptionally regulated to be expressed under iron deficiency, and the monocistronic transcript of *isiA* is more abundant than the dicistronic one [25]. IsiA gene product was found to confer fitness of photosynthetic machinery under iron-limited environments. The product of *isiA* was described in iron-starved *Anacystis nidulans* as an induced chlorophyll-binding protein [26]. This protein was initially named CP43'due to its similarity to CP43, located at the photosystem (PS)II [25]. Initially, IsiA was proposed to play a role as an additional light-harvesting complex [27], and over the years, several functions have been suggested, summarized by Sun and Golbeck [28]: (i) IsiA is a chlorophyll storage protein for the rapid recovery of the cyanobacteria after stress [29]; (ii) it acts as an excitation energy dissipator, protecting PSs from photoinhibition [30]; (iii) it serves as a light-harvesting complex potentially for both PSs [27, 31] and (iv) IsiA replaces CP43 in PSII and permits a cyclic electron transfer pathway involving PSII and the cytochrome b_6f complex [32, 33].

It is interesting to note that *isiA* is not present in all cyanobacteria, and no homologs of *isiA* have been found in plants. In fact, the presence of *isiA* in cyanobacteria found in the iron-limited,

high-nutrient low-chlorophyll regions of the equatorial Pacific lead to the suggestion that the presence of this gene can be a natural biomarker for iron limitation in oceanic environments [34].

In most unicellular cyanobacteria downstream, *isiA* lies the *isiB* gene that encodes a small FMN-flavoprotein called flavodoxin. It is noticeable that, usually, in filamentous cyanobacteria, the flavodoxin gene is transcribed independently of *isiA* and lies in a different locus. Flavodoxin allows that the distribution of light energy as reducing power remains unaltered in iron deficient environments. When iron is not available, the synthesis of the iron-sulfur protein ferredoxin is repressed while flavodoxin is induced. Flavodoxin replaces ferredoxin as an electronic transporter in many of the reactions in which ferredoxin participates [35–39]; surprisingly, flavodoxin is not able to functionally replace heterocyst ferredoxin, even though electron transfer chain to nitrogenase is also an iron-dependent process [35]. Flavodoxin is not exclusive of cyanobacteria, and it may also be present in heterotrophic bacteria as well as in a few cases of algae [40]. Cyanobacteria which lack flavodoxin synthesis capability are particularly affected when iron is scarce, and ferredoxin downregulation under adverse conditions severely compromises survival [41]. Ferredoxin and flavodoxin are isofunctional proteins, but they do not share any significant similarity in primary, secondary or tertiary structures. These proteins can interact productively with the same redox partners [37, 38] and exhibit kinetics constants in the same range even though flavodoxin is slightly less efficient [37].

Flavodoxin expression is induced not only under iron deficiency but also under a wide range of several environmental stresses that result in ferredoxin downregulation [38, 42, 43], especially oxidative stress. Concerning the photosynthesis, flavodoxin behaved as an alternative intermediate for the photosynthetic electron transfer chain *in vivo*, acting, as ferredoxin does, as the main distributor of the reducing power [38, 44]. Under iron limitation, reduced flavodoxin also signals for the whole cell the presence of an active photosynthetic electron transfer chain through the thioredoxin electron transfer pathway. Reduced thioredoxins via thioredoxin reductase, regenerates, through reduction of their cysteine residues, the active forms of many target enzymes as peroxiredoxins, Calvin cycle enzymes and $NADP^+$-malate dehydrogenase, among others. Flavodoxin allows that this key process is still working under iron deficient conditions.

Since flavodoxin synthesis is one of the first responses to iron deficiency [45], flavodoxin was first proposed as an iron-deficiency biomarker in the marine diatom *Thalassiosira weissflogii* [46]. Similarly, in the green algae *Scenedesmus vacuolatus*, the ferredoxin/flavodoxin ratio [47, 48] was used as iron-stress molecular marker.

2.1.2. IdiA, IdiB and IdiC proteins

In cyanobacteria under iron and manganese limitation, the *idiA* gene expresses the iron deficiency-induced protein, IdiA [49]; No counterpart seems to exist in green algae and higher plants [22]. The transcriptional regulator IdiB regulates the expression of *idiA*, in a response controlled by iron availability [50]. IdiA plays an important role in protecting the acceptor side of PSII against oxidative damage, especially under iron-limiting growth conditions [51].

IdiA shows considerable sequence similarity to a family of bacterial periplasmic ABC transporter complexes involved in iron import known as FutA, SfuA, FbpA or HitA (http://genome.

microbedb.jp/cyanobase/). Although some IdiA-similar proteins have been found in the periplasm [52], IdiA is predominantly found associated to thylakoids [53], suggesting different functions for the distinct IdiA-similar proteins [52]. IdiA undergoes prominent structural changes upon iron deficiency and forms a tight and specific complex with dimeric PSII by interaction with CP43 and D1 [54], suggesting that IdiA protects the acceptor side of PSII, which is more exposed under iron limitation due to ongoing phycobilisome degradation [54].

In the *idi* operon, IdiB positively regulates transcription of *idiA* under iron starvation. IdiB encoding a member of the Crp/Fnr transcriptional regulators family [55] is transcribed under iron limitation and oxidative stress and controlled itself by iron-responsive Fur family members [56]. A third iron-regulated gene is *idiC*, belonging to the thioredoxin-like (2Fe–2S) ferredoxin family. Even though IdiC synthesis is constitutive, iron limitation induces a strongly enhanced expression of *idiC*. IdiC is loosely attached to the thylakoid and to other membranes, and its expression is enhanced during conditions of iron starvation or during the late growth phase [57]. Even though its role is still unclear, based on the similarity of IdiC to NuoE of the respiratory *Escherichia coli* NDH-1 complex, it has been suggested that IdiC is a component of the NADH-1 complex in *Synechococcus elongatus* and, thus, has a function in the electron donation from NAD(P)H to plastoquinone. Under stress conditions, when PSII resulted damaged, IdiC would prevent or reduce the oxidative stress deviating electron transport via alternative dehydrogenases, increasing PSI cyclic flow interconnected with respiratory routes [57].

2.2. Siderophore synthesis and induction of high affinity transporters

Derepression or induction of high affinity transporters to enhance iron acquisition as well as siderophore synthesis and cell surface enzymes production is a generalized response to iron starvation [1]. In cyanobacteria, siderophore-mediated iron uptake is thought to be an evolutionary advance that contributes to dominate iron-limited environments. Siderophores are strong Fe^{3+} chelators, and some of them synthetized by nonribosomal peptide synthetase systems. Siderophore production and secretion occurs, especially under iron starvation, when the intracellular iron concentration drops below a certain threshold required for functionality [58]. Siderophore-iron complexes are bound by outer membrane receptor proteins, the TonB-dependent transporters (TBDTs). These outer membrane receptors are generally induced by iron starvation and usually are not present or poorly expressed under iron-sufficient conditions [1]. The iron uptake, transport and storage mechanisms in cyanobacteria are reviewed in detail in Section 3.

2.3. Retrenchment

Retrenchment or downregulation of physiological rates is a progressive and reversible response, resulting in a modulation of the overall growth rate and changes in biochemical parameters. This mechanism is widely used in the adaptation of many organisms to adverse conditions. The most frequent response implies remodeling of bioenergetic pathways in response to iron availability (see Sections 2.1 and 5). As mentioned previously, low iron concentrations trigger a reduction in the level of iron-rich photosynthetic proteins in cyanobacteria while iron-rich mitochondrial proteins are preserved [22].

Cell size reduction and/or morphological changes as response to iron starvation have also been described. For example, thylakoidal membranes and carboxysomes decrease as well as glycogen storage granules increase were observed in *A. nidulans* R2 by electron microscopy [26]. Iron limitation causes morphological changes in the thylakoid packing, promoting unpacking [59]. This phenomenon may be related with phosphorylation of light-harvesting chlorophyll-binding protein of PSII (LCHII) in barley induced by iron deficiency [60]. Iron deficiency causes in cyanobacteria a reduction of cell size [61, 26], sometimes related with growth rate [26, 62].

3. Iron uptake, transport and storage

Siderophores are low-molecular-weight (generally <1000 Da) extracellular iron chelators produced by many prokaryotes and some eukaryotes including fungi, yeasts and plants. These secreted molecules often have a peptidic backbone, with modified amino acid side chains creating three main types of iron-coordinating ligands, that is hydroxamates, catecholates and carboxylates, which commonly form hexadentate octahedral complexes with one ferric ion [63, 64].

Most of the cyanobacterial siderophores appear to contain hydroxamate groups [65, 66], including the dihydroxamate siderophores schizokinen [65, 67] and synechobactin [68], though some species produce catecholate-type chelators such as anachelins [69, 70]. Hydroxamate-based siderophores are strong organic chelators showing a 1:1 stability constant with ferric iron of ~10^{30}, something greater than that of the Fe^{3+}-EDTA complex (~10^{25}); however, ferric-catecholate siderophore complexes almost duplicate this affinity (~10^{49}) [71]. Siderophores may coordinate other metals such as Zn^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+}, Cd^{2+}, Mn^{3+}, Co^{3+}, Al^{3+}, and Cr^{3+}, playing significant roles in the biogeochemical cycling, biological uptake, and protection against deleterious exposure to high concentrations of these elements [72, 73]. In fact, the cyanobacterial siderophore schizokinen binds Cu^{2+} and contributes to alleviate copper toxicity under high environmental copper concentration. Secreted schizokinen sequesters extracellular Cu^{2+}, but cupric-schizokinen is not recognized and internalized by cyanobacterial outer membrane transporters, thereby lowering the amount of copper taken up by the cells [74]. A similar detoxifying effect of cyanobacterial dihydroxamate siderophores has been observed with cadmium [75].

Among freshwater cyanobacteria, the model filamentous nitrogen-fixing heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120 as well as the bloom-forming, toxin-producing *A. flos-aquae* synthesize schizokinen as their major siderophores [76]. Hydroxamate-based siderophore production has also been described in the paddy field cyanobacterium *A. oryzae* [75], and in nontoxic strains of the bloom-forming cyanobacterium *Microcystis aeruginosa* [77]. A novel group of cyanobacterial catecholate-type siderophores known as anachelins has been described in *A. cylindrica* [69]. In marine environments, only the coastal cyanobacterium *Synechococcus* sp. has been reported to produce siderophores. Notably, a distinct suite of dihydroxamate siderophores termed synechobactins is produced by *Synechococcus* sp. PCC 7002 [68]. In addition, xenosiderophore uptake (i.e., aerobactin and desferrioxamine B) has been documented in cyanobacteria [65], though the uptake of self-secreted siderophores is more efficient [78].

The routes of siderophore biosynthesis have not been extensively studied in cyanobacteria. Siderophore biosynthesis occurs in heterotrophic bacteria by two main pathways: one is directed by a large family of modular multienzymes called non-ribosomal peptide synthetases (NRPSs) and polyketide synthetases (PKS), while the other is known as the NRPS-independent siderophore (NIS) pathway [79]. Biosynthesis of hydroxamate-based siderophores with similar structures to schizokinen and synechobactins (e.g., aerobactin) takes place by the second route, involving four enzymes encoded by the gene cluster *iucABCD*, usually organized as an operon [80]. In *Anabaena* sp. PCC 7120, the outer membrane transports for ferric-schizokinen SchT (Alr0397) has been characterized [11], which showed a high amino acid sequence similarity with the ferric-aerobactin IutA transporter from *E. coli*. Near to the gene *alr0397*, a cluster of four open reading frames (*all0394*, *all0393*, *all0392*, *all0390*) show similarity with *iuc* genes, suggesting a role in the biosynthesis of schizokinen [11]. Since the defining characteristic of the NIS biosynthetic pathways is the presence of one or more nucleotide triphosphate-dependent synthetases responsible for condensation reactions during siderophore biosynthesis, this route has also been proposed for hydroxamate-based siderophore biosynthesis in *A. variabilis* and *Synechococcus* sp. PCC 7002 [81, 82].

Another putative route of siderophore biosynthesis in *Anabaena* sp. PCC 7120 occurs presumably via a template-directed, nucleic acid-independent non-ribosomal mechanism which is mediated by the gene products of a cluster of nine open reading frames, from *all2641* to *all2649*, encoding seven NRPSs and two PKSs [83]. The expression of this NRPS-PKS gene cluster is transcriptionally repressed by the master regulator of iron homeostasis FurA [9], being induced under iron limitation or oxidative stress condition [83]. Since iron starvation induces oxidative stress in *Anabaena* sp. [7], maybe by dysfunction of the photosynthetic electron transport and some iron-containing antioxidant enzymes (e.g., SodB and DpsA), it has been postulated that release of siderophore biosynthesis to increase iron uptake during oxidative stress could restore both photosynthesis and ROS scavenging [83]. The protective effect of siderophores against oxidative stress has also been documented in heterotrophic bacteria [73].

De novo synthetized and re-used siderophores are secreted to the outside environment of bacterial cells by export systems which are not very well known in cyanobacteria. In *E. coli*, the export of enterobacterin siderophore involved different mechanisms comprising at least two components, the outer membrane channel tunnel protein TolC [84] which transports the siderophore from the periplasm to the outside, and several inner membrane transporters including the major facilitator superfamily (MFS) protein EntS [85] and the resistance nodulation cell division (RND) transport proteins AcrB, AcrD, AcrEF, MdtABC, and MdtEF [86]. In *Anabaena* sp. PCC 7120, the deletion mutant of the MFS-type inner membrane protein SchE (All4025) failed to secrete schizokinen siderophore to the external milieu [59]. Similar results were observed upon deletion of gene *hgdD* (*alr2887*) [59], encoding the only TolC-like protein in *Anabaena* sp. PCC 7120, termed HgdD, which is also required for protein and glycolipid secretion during heterocyst development [87] and secondary metabolite/antibiotic export [88]. Hence, hydroxamate siderophores appear to be exported in this model cyanobacterium through the mechanism SchE-HgdD.

Once bound to iron, ferric-siderophore complexes are efficiently taken up in Gram-negative bacteria through transport machinery which involves different outer and inner membrane-associated proteins as well as soluble periplasmic binding proteins [1, 12]. First, iron-loaded siderophores are recognized and translocated into the bacterial periplasm by TonB-dependent transporters (TBDTs) located in the outer membrane, in a process that is driven by the cytosolic membrane potential and mediated by the energy-transducing TonB-ExbB-ExbD system. Next, periplasmic binding proteins shuttle ferric-siderophores from the outer membrane transporter to ATP-binding cassette (ABC) permeases associated to the cytoplasmic membrane which delivers the iron-loaded siderophores to the citosol [1].

TBDTs are composed of a transmembrane β-barrel domain that encloses a globular plug domain, and a periplasmic exposed TonB box [89]. Bacteria often possess multiple TBDT receptors, each providing the bacterium with specificity for different siderophores [90], but also allowing uptake of other nutrients [89, 91, 92]. TBDTs involved in iron uptake are generally induced by iron starvation and usually are not present or poorly expressed under iron-sufficient conditions [1]. Twenty-two TBDTs have been identified in the genome sequence of *Anabaena* sp. PCC 7120, most of them integrated into gene clusters or even putative operons containing genes coding for proteins involved in iron transport [93]. A TBDT receptor involved in schizokinen uptake, SchT (Alr0397), has been described in *Anabaena* sp. PCC 7120 [11]. The expression of this outer membrane ferric-siderophore transporter is induced under iron-limitation [11], and it is transcriptionally regulated by FurA [94]. SchT appeared not essential for cyanobacterial growth under iron-limited conditions, suggesting the occurrence of other iron transporters in *Anabaena* sp. [11]. A second TBDT termed IacT (All4026), involved in iron and copper uptake, has been characterized in *Anabaena* sp. PCC 7120. IacT is not a schizokinen transporter; it appears to function under conditions in which the copper concentration exceeds the concentration of iron and seems to transport iron as ferric-citrate [59]. Finally, a third TBDT also involved in ferric-schizokinen uptake, IutA2 (Alr2581), has been recently described [78]. The *iutA2* mutant showed significant growth impairment under iron deprivation as well as alterations in ferric-schizokinen uptake.

Beyond the TBDTs SchT and IutA2, the iron-loaded schizokinen uptake machinery in *Anabaena* sp. PCC 7120 appears to comprise, at least, the gene products of *tonB3* (*all5036*), *exbB3*/*exbD3* (*all5047*, *all5046*), and *fhuCDB* (*all0389-all0387*). Whereas several *tonB*-like genes, *exb* clusters, and permease systems (i.e., *fhu*, *fut*, *fec*) have been annotated in the *Anabaena* genome, only the expression of the abovementioned ORFs were induced under iron-limiting conditions and reduced at high iron concentrations [12]. Additionally, mutants of the periplasmic ferric-siderophore binding protein FecB1 (All2583), but not of its homolog FutA, showed a slightly reduced uptake rate of ferric-schizokinen [78]. The *Anabaena* sp. PCC 7120 siderophore uptake system SchT/FhuBCD appears to be also involved in ferric-aerobactin uptake; however, the uptake of this hydroxamate siderophore produced by *E. coli* was ~10 fold slower than the uptake of ferric-schizokinen in the filamentous cyanobacterium [78].

Whereas some cyanobacterial species produce siderophores to scavenge iron under iron-limiting conditions, many cyanobacteria do not possess this ability, including some environmentally relevant lineages such as the planktonic freshwater cyanobacterium *Synechocystis* sp. [95], the

dominant picocyanobacterium *Prochlorococcus marinus* [82], and the open-ocean nitrogen fixers *Trichodesmium erythraeum* and *Crocosphaera watsonii* [82, 96]. However, some non-siderophore-producing cyanobacteria express all the components of iron-siderophore uptake machinery, being capable of incorporate xenosiderophores [97]. Reductive iron uptake appears extended in many non-siderophore-producing cyanobacteria. In this strategy, reduction of free or complexed ferric iron (e.g., ferric-citrate) into its ferrous form takes place prior to the transport across the plasma membrane either by iron-reducing superoxide radicals secreted to the extracellular milieu as has been described in *Trichodesmium* and *Lyngbya* [96, 98], or through the action of plasma membrane-associated respiratory terminal oxidases as occurs in *Synechocystis* sp. PCC 6803 [95]. Given their small sizes, hydrophilic ferrous and unchelated ferric ions may passively diffuse to the periplasmic space through nonspecific outer membrane porins [95]. However, due to its frequent environmental low concentration, ferric iron uptake usually requires TonB-ExbB-ExbD-dependent active transport systems [99]. Once into the periplasm, high affinity ferric-binding soluble proteins bind ferric ions such as FutA1 and FutA2 and shuttle them to inner membrane ferric permeases such as FutB and FutC [100, 101]. Alternatively, ferric ions are reduced by any of the abovementioned mechanisms and cross the inner membrane through ferrous iron transporters like FeoB [95].

Once inside the cell, ferric iron is reduced to ferrous iron, which has a much lower affinity for the siderophore and spontaneously dissociates [1]. Due to poor bioavailability or iron and its frequent intermittent supply in nature, bacteria have evolved efficient iron storage mechanisms involved ubiquitously multi-subunit proteins termed ferritins and bacterioferritins [102]. These proteins can accommodate up to 4500 iron atoms into a central cavity in a form that is unlike to participate in ROS generation reactions [102, 103]. In *Synechocystis* sp. PCC 6803, bacterioferritins BfrA and BfrB are responsible for the storage up to 50% of intracellular iron content [104], while the DPS family ferritin MrgA plays a pivotal role in both the mobilization of the stored iron within the cell [105], and the coordination between iron homeostasis and oxidative stress response [4]. By contrast, little is known about the mechanisms of iron storage in *Anabaena* species. Only four nonheme-binding ferritin family genes have been identified in *Anabaena* sp. PCC 7120 [104], including *alr3808* [106] and *all1173* [107], encoding two DNA-binding protein homologs to DpsA from *Synechococcus* sp. PCC 7942 [108]. DpsA from *Synechococcus* displays a weak catalase activity *in vitro* and is presumably involved in peroxide-consuming mechanism located on the chromosomal DNA, conferring resistance to peroxide damage during oxidative stress conditions or long-term nutrient limitation [108]. According to the CyanoBase [109], the genomes of other environmentally relevant cyanobacteria such as *P. marinus*, *C. watsonii*, *T. erythraeum*, and *M. aeruginosa* encode members of the ferritin/bacterioferritin superfamily.

4. Regulation of iron homeostasis

Regulators of the Fur (Ferric uptake regulator) family constitute the primary mechanism in the maintenance of iron homeostasis in cyanobacteria. The first evidence of the existence of a Fur protein in cyanobacteria was the isolation of a *fur* gene in *Synechococcus* PCC 7942 through an *E. coli*-based *in vivo* repression assay [13]. Apart from *Synechococcus*, Fur homologs

have been mainly identified and studied in *Synechocystis*, *Anabaena* and *Microcystis* [17, 110–112]. Cyanobacterial Fur proteins contain histidine rich motifs (HHXHXXCXXC) as potential metal binding sites, which share properties with Fur from other prokaryotes [113, 114]. In the classic model of operation for this transcriptional regulator, Fur functions as a repressor, using ferrous iron as a co-repressor. Under sufficient iron availability, a dimer of active Fe^{2+}-Fur complex binds to *cis* regulatory elements in the promoter of target genes and thereby prevents transcription [115]. However, other regulatory mechanisms have been described indicating that Fur can also bind to specific promoters in its apo form repressing transcription. Even apo- and holo-Fur activations have been reported [113, 116]. In the cyanobacterial genomes, it is common to find diverse ORFs that encode different Fur homologs which perform several functions. In this sense, in *Synechococcus* 7002 or *Anabaena* sp. PCC 7120, three *fur*-type genes exist, but only one of them, denoted as *furA*, appears directly involved in upregulation of iron uptake genes under iron limitation [9, 117, 118]. Recent studies confirmed that FurA is an essential, well-conserved protein among cyanobacteria. A significant depletion of *furA* expression levels impaired the photoautotrophic growth of *Anabaena* sp. under standard culture conditions in both, solid and liquid media [14]. FurA is the master regulator of iron homeostasis in *Anabaena* sp. PCC 7120 [9] and presumably in many other cyanobacterial species [14]. FurA modulates not only the expression of the iron metabolism machinery, but also regulates directly or indirectly the transcription of a plethora of genes and operons involved in a variety of physiological processes including photosynthesis, respiration, response to oxidative stress, nitrogen fixation, heterocyst differentiation, cellular morphology, tetrapyrrole biosynthesis pathway, phycobilisome degradation, chlorophyll catabolism, programmed cell death, light sensing and response, signal transduction systems, exopolysaccharide biosynthesis, and cyanotoxin production, among others [15, 16, 94, 119].

Cyanobacterial Fur regulators can function both as activator and repressor as observed in the transcriptional regulation by FurA of genes involved in the tetrapyrrole biosynthesis pathway in *Anabaena* sp. PCC 7120 [9]. In all these cases, regulation by Fur adapts the answer to provide iron in case of deficiency of this metal or to allow its storage or the use of proteins that depend on iron when this metal is sufficient [1]. Fur recognizes AT rich regions called Fur boxes located in the promoter region of iron responsive genes [120]. Although it is assumed that this regulator binds as a dimer to the promoter, a computational study of Fur proteins from *Synechocystis* sp. PCC 6803 proposed the binding of multimers of the Fur-like regulator onto its target DNA, which possesses internal repeats [121]. Lately, atomic force microscopy revealed the sequential binding of FurA to its own promoter boosted by DNA bending in *Anabaena* sp. PCC 7120 [122]. Cyanobacterial Fur-DNA recognition depends not only on metal levels. Apart from iron, a reduced form of FurA from *Anabaena* sp. PCC 7120 is required for *in vitro* optimal DNA-binding [112, 123]. Also, reduction of Fur from *M. aeruginosa* PCC 7806 increases the binding affinity to its target genes [124]. Cyanobacterial Fur homologs contain a variable number of cysteine residues in their primary sequence and the need for reducing power for this regulator to develop its function is based on the importance of the redox state of these residues. A cysteine mutational study of the five cysteines present in *Anabaena* sp. PCC 7120 Fur sequence revealed that C^{101}, a residue conserved in most bacterial

Fur homologs, is part of a thiol/disulfide redox switch that determines FurA ability to bind the metal co-repressor [125]. Moreover, this residue belongs to a CXXC motif responsible of the disulfide reductase activity exhibited by *Anabaena* FurA, suggesting that Fur is involved in the cyanobacterial redox-signaling pathway. Apparently, Fur connects the response to changes in the intracellular redox state and iron management in cyanobacteria [126].

The amount of Fur is controlled in cyanobacteria by mechanisms present in the three levels of the flow of genetic information [123]. At the transcriptional level, the TetR family transcriptional regulator PfsR regulates *fur* transcription in *Synechocystis* PCC 6803. A *pfsR* deletion mutant displayed stronger tolerance to iron-limiting conditions as compared with the wild type. Moreover, the transcripts of *pfsR* were enhanced by iron limitation and inactivation of the gene affected pronouncedly expression of *furA* gene and genes involved in iron transport and storage among others [127].

At the post-transcriptional level, *cis*-encoded antisense RNAs regulate Fur expression in cyanobacteria [128]. In *Anabaena* sp. PCC 7120, a large dicistronic transcript encoding the putative membrane protein Alr1690 and a *α-furA* RNA transcript complementary to *furA* is involved in the control of the cellular levels of the protein [129]. Also, *cis α-furA* RNAs are present in *M. aeruginosa* PCC 7806 and *Synechocystis* sp. PCC 6803 [130].

Regulation of the Fur level and its activity also take place post-translationally by different mechanisms in cyanobacteria. It has been reported that the membrane cytoplasmic FtsH1/FtsH3 protease heterocomplex, involved in the acclimation of cells to iron deficiency, controls the availability of *Synechocystis* sp. PCC 6803 Fur by degradation of apo-Fur in order to regulate transcription of iron responsive genes [131]. Moreover, cyanobacterial Fur can form a complex with heme that alters its ability to join to DNA. In particular, *Anabaena* sp. PCC 7120 FurA interacts strongly with heme in the micromolar range of concentration and inhibits the *in vitro* ability of this protein to bind to DNA [117]. The axial ligand of heme in the FurA-heme complex is a cysteine residue that belongs to a Cys-Pro motif (Heme regulatory motif) present in its primary sequence and the sequences of all cyanobacterial homologs but absent in most non cyanobacterial ones. The regulator undergoes a redox-dependent ligand switch so that heme could be involved in sensing redox variations within the cyanobacterial filament and alter the regulatory function of FurA [132].

A novel layer of complexity of iron homeostasis regulation in cyanobacteria involves RNA molecules as IsaR1. When iron is scarce, IsaR1 affects the photosynthetic apparatus in three different ways: (1) directly, inhibiting the expression of proteins important in photosynthesis; (2) indirectly, by suppression of pigment production; (3) preventing the expression of proteins that contain iron-sulfur clusters. Homologs of IsaR1 are conserved throughout the cyanobacterial phylum [133]. Also, the SufA and IscA proteins, proposed to function as scaffolds in the assembly of Fe/S clusters in bacteria, seem to play regulatory roles in iron homeostasis in cyanobacteria, according to experiments performed on single and double null-mutant strains of *Synechococcus* sp. [134]. Even the three PchR regulators (PchR1, PchR2, PchR3) present in *Synechocystis* PCC 6803 seem to play a prominent role in the protection against iron stress, among other stresses, in this cyanobacterium [135].

5. The regulation of iron homeostasis is tightly connected to central metabolic pathways

As mentioned previously, iron deficiency is one of the major causes of stress in cyanobacterial communities. Due to the occurrence of iron in most electron transport proteins conforming photosynthetic, respiratory and nitrogenase pathways, the adaptive strategies developed by the cyanobacteria are tightly related to the rearrangement and modulation of these processes. Furthermore, many of the different responses triggered by iron deprivation are aimed to prevent and alleviate oxidative stress and to the modulation of central metabolism.

5.1. Iron availability and the oxidative stress response

Oxidative stress is one of the many consequences of iron imbalance in cyanobacteria. Thus, the control of iron homeostasis is intimately linked to the regulation of many genes involved in the response to oxidative stress [4, 14, 24, 94]. Moreover, the master regulators involved in such processes in cyanobacteria, namely FurA and PerR/FurC, display a set of common targets [14, 136]. Furthermore, PerR/FurC is able to modulate *in vitro* FurA-DNA binding activity [117]. Transcriptomic analyses and differential proteomics focused on the definition of the FurA regulon in *Anabaena* PCC 7120 unveiled that around 13% of FurA targets with a known function were involved in detoxification of ROS [14]. Those FurA-regulated genes belong to different subcategories, such as electron transport proteins dedicated to restore oxidized thiols (*trxA*, *trxB*, the glutaredoxin-related protein *alr0799* and the glutathione S-transferases *alr3195* and *alr7354*, among others); detoxification of hydrogen peroxide (the Mn-catalase *katB* and the peroxiredoxins *all1541* and *alr4641*) or the protection of DNA (*dpsA*) [14, 106, 119, 137]. FurA also controls the expression of flavodoxin that is strongly induced under iron deficiency [13, 138]. Initially described as a substitute for ferredoxin I (Fd) in the photosynthetic electron transport to $NADP^+$ [45, 138] (reviewed in Sections 2.1.1 and 5.2), flavodoxin is also a powerful scavenger of ROS. Interestingly, the expression of flavodoxin in chloroplasts of tobacco unveiled that this flavoprotein is able to effectively interact with several Fd-dependent oxidoreductive pathways, including thioredoxin reduction [139]. The expression of flavodoxin in plastids protected target enzymes of central metabolic pathways from oxidative inactivation, such as the Calvin cycle components fructose-1,6-bisphosphatase (FBPase) and phosphoribulokinase (PRK). Therefore, the expression of flavodoxin triggered by iron deficiency relieves the oxidative stress in the cyanobacteria and contributes to the reconstitution of the electron transport chains rich in iron-containing proteins whose iron-sulfur clusters are immediate targets of free radicals, minimizing the effect of the oxidative damage on the photosynthetic rates and the nitrogen metabolism, among other metabolic pathways [139].

5.2. Influence of iron availability in the control of photosynthetic genes

As it has been shown previously, iron limitation has important consequences in the composition and performance of cyanobacterial photosystems. Several photosynthetic cyanobacterial specific genes induced under iron deficiency contribute to modify their photosynthetic machineries such as *isiA*, *isiB* (flavodoxin), *idiA*, *idiB* and *idiC* proteins (reviewed in Section 2.1.1). Fur controls the expression of *isiA* and isiB [13], whose transcription is induced by multiple stresses such as treatment with hydrogen peroxide or high salt [56, 136, 140].

Further transcriptomic studies evaluating the cyanobacterial response to iron deficiency unveiled that as a general trend, photosynthesis genes were repressed under low-iron conditions and induced upon the re-addition of iron. Many of those genes belonged to the *psa* and *psb* families, components of the phycobilisomes and genes involved in the synthesis of chlorophyll are also direct targets of FurA [14, 24, 141]. Furthermore, Fur is involved in the control of genes involved in carboxysome formation and Calvin cycle. Notably, a close relationship between light availability and iron requirements can be inferred from different studies, such as the differentially expressed genes in [142], the regulation of *furA* and the *alpha-furA* antisense RNA by light [143], or the need of an active photosynthetic electron transport chain for the expression of the *mcy* operon in *M. aeruginosa*, that in turn is controlled by FurA [124, 143, 144]. As *furA* from *M. aeruginosa*, the expression of the *Anabaena* sp. PCC7120 ortholog is controlled by an antisense RNA whose inactivation produces iron-deficient cells and severe structural disorders in the photosynthetic apparatus of *Anabaena*. Furthermore, disruption of the dicistronic message encoding the *alr1690-alpha-furA* tandem leads to lower photosynthetic performance indexes, unveiling that its expression is required for maintenance of a proper thylakoid arrangement, efficient regulation of iron uptake and optimal yield of the photosynthetic machinery [123, 145]. In addition, FurA modulates the transcription of the LexA regulator in *Anabaena* PCC7120. This regulator is critical to the survival of cyanobacterial cells facing inorganic carbon starvation, since most of the LexA-responsive genes were known to be involved in carbon assimilation or controlled by carbon availability [146].

5.3. Iron-responsive genes involved in cyanobacterial respiratory pathways

In addition to the photosynthetic electron transport chains, cyanobacterial thylakoids contain multiple respiratory electron transport complexes [147]. Thus, photosynthesis and respiration are tightly related in cyanobacteria since both pathways share several components, such as a quinone/quinol pool [148], plastoquinone, cytochrome b6f and plastocyanin/cytochrome [148, 149]. Furthermore, the cyanobacteria contain a second complete respiratory chain present in the cell membrane that also uses the same mobile quinone pool mediating electrons in the photosynthetic and thylakoidal respiratory processes. Several studies evidence the relationship between the iron pool and the respiratory activity. The major oxidase in cyanobacteria, COX, is encoded by the *cox* operon (*coxBAC*) and FurA regulated though the modulation of *cox*B [15]. Similarly, the transcription of *alr0869* (*ndhF*) and the subunit 5 of NADH dehydrogenase encoded by *all1127* are regulated by FurA as response to iron availability [15]. Furthermore, iron starvation in *S. elongatus* causes upregulation of several cytochrome oxidases and the increase of respiratory electron transport [22, 150], while an *Anabaena* mutant lacking of the *alr1690-alpha-furA* message that exhibits a reduced iron pool with respect to the wild-type strain has affected its respiratory activity [145].

5.4. Cross-talk between iron and nitrogen metabolism

The electron carriers involved in nitrogen metabolism are also rich in iron, especially the proteins involved in nitrogen fixation. Nitrogenase and nitrogenase reductase complex harbor around 40 atoms of Fe^{2+} distributed between the iron-molybdenum cofactor (FeMo-co) and the [8Fe-7S] P-cluster present in NifDK nitrogenase, and the [4Fe-4S] cubane in the NifH dinitrogenase reductase. In addition, most of the proteins involved in the assembly

of the metalloclusters embedded within the NifDK protein also contain diverse [Fe-S] centers [151, 152]. Thus, growing under nitrogen fixation conditions adds an additional iron stress to the cell. Therefore, optimal cyanobacterial performance requires a tight and coordinated regulation of iron and nitrogen metabolisms [137]. Nitrogen metabolism in cyanobacteria is controlled by the master regulator NtcA [153] that usually senses the C/N balance through the intracellular 2-oxoglutarate levels [154]. NtcA controls a wide regulon of genes involved in different functional categories [155, 156]. Among them, NtcA controls most steps required for nitrogen fixation in cyanobacteria, starting from heterocyst differentiation and development until *nif* genes expression. NtcA also controls key genes in nitrogen assimilation pathways in cyanobacteria [157]. Different studies evidence a tight relationship between iron and nitrogen metabolism. Interestingly, transcription of the *nifHDK* operon and excision of the 11 kb DNA fragment required for heterocyst differentiation was observed in iron-starved *Anabaena*, even though cells grew in the presence of combined nitrogen [138]. Further studies showed that the expression of FurA is highly induced in the heterocyst [137]. FurA participates in the regulation of *nif* genes, and the levels of this regulator are critical for the modulation of heterocyst differentiation by controlling the expression of NtcA and vice versa [14, 16]. Thus, several iron-responsive genes in cyanobacteria, such as *nblA*, *petH*, *pkn41*, *pkn42*, among others, are also modulated by NtcA [137, 158–161]. Conversely, in *Synechocystis sp.* PCC 6803, the NtcA-regulated genes *bgtB*, *glnA* and *urtB* are highly upregulated under iron limitation [162]. Different studies focused on the identification of the FurA and NtcA regulons in different cyanobacterial strains support that FurA and NtcA are interactive regulators and corroborate that both transcription factors share an important number of targets mainly related to photosynthesis and respiration, iron uptake and incorporation, oxidative stress response and nitrogen metabolism [137]. However, given that both FurA and NtcA are global regulators, it is not surprising that the nitrogen starvation response involves a large number of genes not only related to iron metabolism but also to heavy metal and oxidative stress adaptation, reinforcing the interrelationship of those processes [162].

6. Iron involvement in cyanotoxin production

Metabolic plasticity of cyanobacteria includes the synthesis of a broad variety of secondary metabolites, some of them potentially toxic for eukaryotic organisms, the so-called cyanotoxins [163]. When toxins are synthetized, the cyanobacteria divert large amounts of carbon and nitrogen to this process so that it might be obvious to think that cyanotoxin synthesis gives them some adaptive advantage. Cyanotoxin production is not universal or constant even among those species and strains holding the necessary genes. The conditions that induce cyanotoxin production in capable species have not been elucidated. Under certain environmental conditions, cyanobacteria can proliferate to form blooms consisting of significant biomass and covering large areas in fresh or marine water. It is necessary to separate the phenomenon of blooms occurrence from the fact of toxicity, although obviously the problem is detected when the population of toxic cyanobacteria synthetizing toxins is high.

6.1. Iron and blooms occurrence

Iron availability and biolimitation by iron of the phytoplankton are important subjects discussed for many years. After IronExII [2], it was definitively established that iron availability limits rates of cell division, as well as abundance and production of phytoplankton of the equatorial Pacific and likely in other "high nutrient, low chlorophyll regions" [55]. There is broad agreement that nutrient over-enrichment of freshwater and marine ecosystems promote cyanobacterial blooms. Phosphorus and nitrogen have traditionally been considered the key nutrients limiting primary productivity and algal biomass. But based on such accessibility (and light and temperature suitable for cyanobacterial growth), iron availability could be suggested to be the switch that triggers a bloom. Cyanobacteria compete very efficiently with other phytoplankton species for iron resources and often end up dominating the population. In addition to all, the adaptive strategies previously mentioned, in some cases, their competitive advantage is based on its ability to vertical migration [164].

6.2. Iron and cyanotoxin production

Cyanotoxins are a heterogenous group of molecules that include hepatotoxins, neurotoxins, dermatotoxins and cytotoxins, with diverse chemical nature such as cyclic peptides: cyclic peptides, alkaloids, non-proteic amino acids. The synthesis of most toxins is inducible, and the genes involved in its biosynthesis have been identified during these last years [165]. The genes conforming biosynthetic pathways, its regulation and the molecular mechanisms involved in toxicity are in each case different. However, NRPS are present in all the described toxic operons, involved in cyanotoxin synthesis. Many NRPS present in many bacteria are iron regulated [166, 167]. A substantial variety of siderophore structures, toxins and antimicrobial molecules with toxic effects are produced from similar NRPS assembly lines [167], and a large number of secondary metabolites are also synthesized as response to iron starvation.

Among cyanotoxins, microcystins are the most ubiquitous toxins causing several environmental and health problems. They are a family of cyclic heptapeptides, synthesized by a mixed PKS-NRPS system called microcystin synthetase encoded in *mcy* operon [168]. The role of microcystins in cyanobacteria is still unclear, but there are evidences that could confer to the toxic strains advantages for survival in iron-limited conditions. The microcystin synthesis has been linked to iron metabolism for many years. Lyck and colleagues [169] showed that during iron depletion, toxic strains of *Microcystis* maintained cell vitality much longer than the nontoxic strains. Moreover, Utkilen and Gjolme [170] found that toxic strains exhibited higher rates of iron uptake than nontoxic strains. They proposed that microcystin could be an intracellular chelator of Fe^{+2}, as well as predicted that the synthesis of the toxin would be controlled by the amount of free iron present in the cells. Structural similarities between microcystin and bacterial siderophores [167] led also to propose a putative role as an extracellular iron-scavenging molecule. Recently, it was shown that while the microcystin producing strain *M. aeruginosa* PCC 7806 and its close strain, the non-producing *M. aeruginosa* PCC 7005 grew similarly in BG11 in the presence of 17 μM iron, under severe iron deficient conditions (0.05 μM), the toxigenic strain grew slightly less than in iron-replete conditions, while the non-producing microcystin strain was not able to grow [171]. Taking together all these data suggest that microcystin production

could be another mechanism evolved by cyanobacteria related to iron homeostasis, on track to survive in iron-limited conditions. In agreement with this statement, it was shown that in *M. aeruginosa* PCC 7806, the *mcy* operon was regulated by Fur [124], and that the *mcy* operon transcription as well as microcystin content were enhanced under iron-limited conditions [172].

Recently, microcystin ability to bind iron and other metals has been demonstrated using various experimental approaches [171], corroborating a possible role of this molecule in iron metabolism. A putative role of microcystin acting as iron chelator involved in iron acquisition has been recurrently suggested. The main problem associated to this theory is the fact that microcystin seems to be an endotoxin although the results showed in bibliography are contradictory. When radioactive inorganic carbon is supplied to *M. aeruginosa* and the fate of intracellular microcystin pool is followed, no export of microcystin was observed [173]. However, the *mcyH* gene included in the *mcy* operon encoded an ABC transporter reported to be essential for microcystin synthesis, suggesting a possible export of microcystin outside of the cell [174]. On the other hand, electron microscopy of immuno-gold labeled microcystin showed that the vast majority of intracellular microcystin is located around the thylakoids [175–177]; hence, a possible role in protecting the photosynthetic machinery to photo-oxidation has been proposed. Recently, it has been described that microcystin can perform metal-driven oligomerization. Some environmental stresses such as low iron or high light conditions cause oxidative stress in the cell which triggers photo-oxidation phenomena. In this scenario, the PSs can be disassembly and then, microcystin could perform oligomerization and capture of iron avoiding metal-dependent Fenton reactions [171]. Another proposed role is related with colony formation performed by *Microcystis* cells. Solid evidences linking microcystin presence and enhanced colony formation and size have been reported [178].

7. Conclusion

Iron is at the core of cyanobacterial metabolic and regulatory networks, playing a central role in the control of electron delivery and distribution in the photosynthetic and respiratory electron transport chains, the reduction of nitrogenase and central metabolic pathways. The adaptive responses of cyanobacteria to iron limitation affect all those processes, though the iron demand of the cell is subject to a hierarchy in favor of photosynthesis. The high quota of iron in cyanobacteria, its ability to promote oxidative stress and its ubiquity in electron transport pathways require a tight control of iron homeostasis mainly performed by FurA. In order to optimize iron resources, the regulation of FurA activity and expression, as well as the genes composing the FurA regulon are strongly interconnected with other master regulators such as PerR and NtcA.

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Conflict of interest

The authors declare no conflicts of interest.

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Cyanobacteria are photosynthetic microorganisms that inhabit almost all geographic locations, including oceans, caves, ponds, lakes, soil, snow, and hot springs. Their acumen to survive and succeed in a plethora of ambiance is due to their extraordinary metabolism and adaptations. Their wide applications include therapeutics, cosmeceuticals, wastewater remediation, biofuels, antioxidative enzymes, agriculture, and so on. This book highlights the important aspects pertinent to the fascinating world of cyanobacteria covering diverse topics such as electron microscopic dimension, cultivation, stress, and adaptations. This book provides insights into the world of tiny microbial factories and unravels the potentials for their futuristic innovative applications as precursors of drug molecules, eco-friendly renewable energy sources, remediation, and reservoirs of diverse value-added products for a better and brighter tomorrow.

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