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Alteration of Cylindrospermopsin Content of *Aphanizomenon ovalisporum* (Cyanobacteria, Nostocales) due to Step-Down from Combined Nitrogen to Dinitrogen

Gábor Vasas[1*], Gyula Surányi[1], István Bácsi[2], Márta M-Hamvas[1], Csaba Máthé[1], Sándor Gonda[1], George Borbely[1]

[1]Department of Botany, Faculty of Science, University of Debrecen, Debrecen, Hungary
[2]Department of Hidrobiology, Faculty of Science, University of Debrecen, Debrecen, Hungary
Email: *vasas.gabor@science.unideb.hu

ABSTRACT

In this study we show that cylindrospermopsin (a cyanotoxin) content of filaments of *Aphanizomenon ovalisporum* ILC164 depended on growth on combined nitrogen or nitrogen fixation. Our results also demonstrated that the shift down of cyanobacterial filaments from combined nitrogen to dinitrogen fixing condition resulted in a significant decrease of cylindrospermopsin pool size which resumed a growth rate dependent manner as the heterocyst and nitrogenase formation appeared. The current study indicated that alteration of nitrogen metabolism of *Aphanizomenon ovalisporum* (Forti) induced changes in cyanotoxin (cylindrospermopsin) metabolism. In addition, this is the first report that isolated heterocysts, the differentiated anaerobic cells for nitrogen fixation of cyanobacteria, did not contain cylindrospermopsin.

Keywords: Cylindrospermopsin; Cyanobacteria; Heterocyst; Nitrogen Starvation; Nitrogenase; Cyanotoxin

1. Introduction

Cyanobacteria, free living obligate photoautotrophic microorganisms can drive energy from sunlight and carbon from carbon dioxide content of the air which dissolves in water. Some cyanobacteria are also able to fix atmospheric dinitrogen. Filamentous nitrogen fixing cyanobacteria that combine aerobic metabolism in their vegetative cells, with anaerobic metabolism in their differentiated cells, called heterocysts are widespread in many eutrophic ecosystems [1-7]. In addition, phytoplanktonic cyanobacteria are responsible for extensive blooms in natural surface waters. Bloom-forming cyanobacteria, among those the nitrogen fixing ones, have caused increasing concern over the last two decades in fresh water and marine environment, since a number of species are capable of producing metabolites that act as toxins of biological origin [8,9,10]. Harmful metabolites of cyanobacteria, called cyanotoxins are a diverse group of natural toxins both from the toxicological and chemical points of view. These toxins can be classified into several categories based on their mode of action. They are neurotoxins, hepatotoxins, harmful lipopolysaccharides, gastroenteral toxins and a variety of other, as yet unidentified toxic compounds [10-14]. In more recent years cylindrospermopsin (CYN), a ulphur containing hepatotoxin (tricyclic guanidyl hydroxymethyluracil) known to produced by *Cylindrospermopsis raciborskii* was concerned in the worst known case of human poisoning in Australia [12,15]. Beside that CYN is produced by several other organisms as well, namely by *Anabaena bergii, Aphanizomenon ovalisporum, A. flos-aquae, Raphidiopsis curvata, Umezakia natans* [9,14-16].

The growth of cyanobacteria, like other organisms, is greatly affected by the concentration of nutrients of which there is often a short supply in natural environment [17]. These organisms are often subjected to limitation in phosphorous, combined nitrogen, sulphur etc. that may give rise to specific alteration of cyanobacterial phy-

siology and metabolism. The response of obligate photo-autotrophic cyanobacteria to changes in nutritional conditions is an active research topic [17,18]. However, there is still a need for studies about the interference of main nutrient limitation with cyanotoxin production of cyanobacteria, especially in the case of CYN. Recently, we have found that sulphate and phosphate starvation of *A. ovalisporum*, a nitrogen fixing and toxic, CYN producing cyanobacterium results in a characteristic reduction of CYN pool size on the basis of cell number and dry mass of culture [19]. However, there are no data on the interference of combined nitrogen starvation with cyanotoxin content of *A. ovalisporum*. In addition, under combined nitrogen limitation, the nitrogen fixing filamentous cyanobacteria induce the differentiation of heterocysts, specialized cells for dinitrogen fixation [2-6,20]. Since nitrogen fixing cyanobacteria are frequently well-known members of harmful blooms in natural waters, it deserves considerable attention to see the alteration of CYN content of cells shifted down from combined nitrogen growth condition to nitrogen fixing one. What is more, no cyanotoxin analysis, including CYN has been reported from heterocysts so far.

2. Materials and Methods

2.1. Strain and Culture Conditions

Cylindrospermopsin producing *Aphanizomenon ovalisporum* strain ILC-164 from Lake Kinneret, Israel was grown in liquid medium of Allen as described earlier [19]. The cultures were kept in glass flasks thermostatically maintained at 28°C and illuminated with cool white fluorescent light (80 µmol·photons·m^{-2}·s^{-1}). Aeration and mixing was achieved by bubbling with sterile air. Growth of the cultures was monitored by measurement of chlorophyll-a content (Chl-a µg·ml^{-1}) in 80 % acetone extracts, cell number and dry mass content. Cell number and heterocyst frequency was determined in 5 µl culture samples in triplicate (all cells of filaments were counted microscopically [19]. For dry mass measurement 3 ml samples were centrifuged (6.000 × g, 5 min), washed and the pellets were lyophilized, weighted and cell mass (mg·ml^{-1}) calculated. All measurements were in triplicates [19].

2.2. Nitrogen Starvation of *A. ovalisporum* Cultures

Combined nitrogen grown exponential *A. ovalisporum* cultures were centrifuged (6.000 × g for 10 min, Beckman Avanti TM Centrifuge, J-25, at 25°C) and the cell pellet was carefully washed twice with sterile combined nitrogen free medium. At zero time the washed cells were resuspended in sterile prewarmed full medium (control) at the same densities or in medium containing only 0.1 mM of combined nitrogen (NH$_4$NO$_3$) for shifting to dinitrogen fixation. The resuspended cultures were further cultivated under otherwise unchanged conditions. Samples were collected each day and used for assays as described above.

2.3. Isolation of Heterocysts from *Aphanizomenon ovalisporum*

Heterocysts were isolated by the lysozyme method of Fay [21,22]. Harvested filaments (centrifuged by 6.000 × g for 5 min) of a 10 l culture fixing nitrogen for 336 h were washed free from culture medium and resuspended in 1 mM Tris-HCl (pH 7.6), 2 mM EDTA, 0.5 M mannitol. The filaments were maintained at 35°C and lysozyme (Sigma) was added at a concentration of 1.0 mg·ml^{-1} and incubated for 45 min. The suspension was put on ice and drawn through a Pasteur pipette. Then suspension was diluted 20-fold with mannitol-Tris buffer (without lysozyme and EDTA) and vigorously vortexed. Heterocysts were collected by differential centrifugation until no vegetative cells were apparent in the pellet (dry mass of vegetative cell free, isolated heterocysts was 127 mg from a 10 l culture).

2.4. Western Blot Analysis

For detection of dinitrogenase reductase protein in nitrogenase complex of cyanobacterial samples, proteins of cell free extracts of *A. ovalisporum* filaments (grown in presence or absence of combined nitrogen) were separated by 7.5% SDS polyacrylamide gel-electrophoresis and the gels were stained with Coomassie Brilliant Blue [23]. The molecular mass of sample proteins was calibrated with the help of SDS-PAGE markers (Pharmacia, LMW Kit). Western blots were carried out after SDS-PAGE to detect presence of dinitrogenase reductase in samples by a modified method of [24]. Briefly, the separated proteins (unstained gels) were transferred to nitrocellulose filter (Protran BA85, Schleicher and Schuell, Germany). Following protein transfer to nitrocellulose (300 mA, 3 hours, Tris-Glycine-Methanol) the membranes were incubated in blocking buffer (phosphate buffered saline /PBS/, pH 7.4 containing 2% bovine serum albumin and 0.1% Tween 20; 1 hour, room temperature). The blot was immunologically probed with a specific antibody raised to dinitrogenase reductase subunit (Chicken anti nifH, Agrisera AB, Sweden). Primary antibody was incubated with the membrane for 7 hours, at room temperature (the primary antibody was diluted 1:3000 in blocking buffer). A blotting grade secondary antibody enzyme conjugate (anti Chicken IgG Alkaline Phosphatase conjugate, Sigma) diluted 1:7000 in blocking buffer was added to bind to the primary antibody (2 hours, room temperature). The alkaline phosphatase

catalyzed the formation of a stable and detectable purple product at the site of dinitrogenase reductase–anti nifH complex from the soluble substrate system 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Sigma) and nitroblue tetrazolium (NBT, Sigma). The working buffer for BCIP--NBT substrate solution was 0.1 M Tris-HCl (pH 9.5) containing 0.5 mM $MgCl_2$. After antibody incubations (primary and secondary antibodies) the membranes were washed 3 times in PBS containing 0.1% Tween 20.

2.5. Amplification of PS Gene and PKS Gene in Vegetative Cells and Heterocysts of *A. ovalisporum*

DNA extraction was achieved by the phenol-chloroform method of [25,26]. PCR was used to analyse the presence or absence of the PKS and PS specific gene region, which are important parts of the gene cluster responsible for CYN biosynthesis. The photo preparation and the analysis of results was achieved using Cleaver Geldoc system. For the PS and PKS gene amplification, the PCR reaction was performed in 25 μl containing 12.5 master mix (Fermentas; PCR buffer, 0.05 u/μl taq Polimerase, 4 mM $MgCl_2$, 0.4 nM dntP), forward and reverse primer, DNA template (100 ng). Amplification was performed in a T Gradient thermocycler, consisting of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 0.5 min at 94°C, 0.5 min at 5°C and 2 min at 72°C and a final extension step of 7 min at 72°C. For the PKS gene amplification, the PCR reaction was performed as above, using PKS M4

(5'-GAAGCTCTGGAATCCGGTAA-3') and PKS M5 (5-AATCCTTACGGGATCCGGTGC-3) primers. For the PS gene amplification, the PCR reaction was performed as above, using PS M13

(5'-GAAGCTCTGGAATCCGGTAA-3') and Ps M14 (5-AATCCTTACGGGATCCGGTGC-3) primers [26]. The PCR products were electrophoresed in 1% agarose gel.

2.6. Analysis of CYN Content of Combined Nitrogen Grown and Nitrogen Fixing Cells and Heterocysts of *A. ovalisporum*

3 ml culture samples were centrifuged (6.000 g for 3 min) and the pellets were lyophilized and used for CYN assay [27,28]. For CYN content assay of heterocysts, isolated heterocysts were resuspended in 1 mM Tris-HCl pH 7.6 buffer and sonicated for 2 min in 15 s intervals on ice at full power. An aliquot of the sonicated material was lyophilized for dry mass assay. The rest of sonicated extract was centrifuged (10.000 g, 30 min, 4°C) and the supernatant assayed for CYN content and a part of it saved for Western blotting. The cyanotoxin content was determined with the help of a slightly modified method of capillary electrophoresis as described earlier by our laboratory

[27,28]. A longer capillary (85 cm) was used to obtain better separation of the compounds. The data were normalized to the zero time control which was chosen to 100 % on basis of cell number.

3. Results

3.1. Growth and Heterocyst Development of *Aphanizomenon ovalisporum* Cultures

Growth curves (cell number·ml^{-1}) from representative experiments of combined nitrogen starved *A. ovalisporum* cultures are shown in **Figure 1**. It is obvious, the
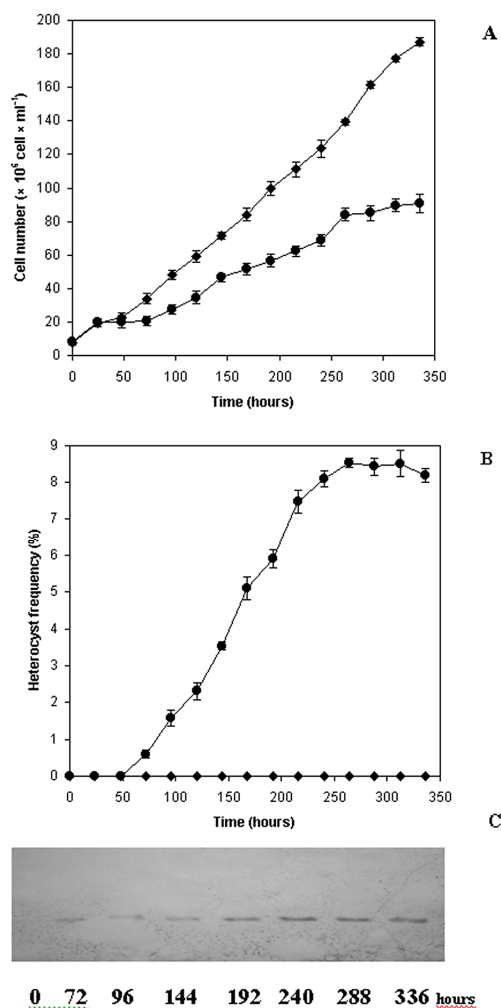

Figure 1. Growth of *Aphanizomenon ovalisporum* cultures on combined nitrogen step-down. An exponential phase culture grown on Allen medium was centrifuged, gently washed with combined nitrogen free medium and divided into two parts (zero time) and further cultivated in full medium (control ―♦―) or starved for combined nitrogen (―●―) throughout the experiment as described in Materials and Methods (A). Alterations of heterocyst frequency (B) and nitrogenase (C) Western blot of *Aphanizomenon ovalisporum* under control (combined nitrogen ―♦―) and nitrogen starvation (―●―) conditions.

combined nitrogen starvation, the turn on dinitrogen fixation reduced the growth rate of the culture (**Figure 1(A)**). When heterocyst development was initiated by replacing combined nitrogen containing medium with minimal medium, growth ceased after 25 hours and recommenced 75 h later. Under the experimental condition described, the removal of combined nitrogen from a culture of *A. ovalisporum* caused the differentiation of a proportion of vegetative cells and resulted in the formation of heterocysts. The mature heterocyst frequency reached a maximum (8.5%) at 250 h (**Figure 1(B)**) and was concomitant with the dinitrogenase reductase subunit level. Consequently, nitrogenase activity developed parallel with the heterocyst formation as shown by western blots (**Figure 1(C)**). The presence of heterocysts and dinitrogenase reductase first became detectable after 60 and 72 hours, respectively (**Figures 1(B) and (C)**). The heterocyst count and the amount of dinitrogenase reductase then rose rapidly and the maximum nitrogenase level recorded 240 h after combined nitrogen starvation (**Figure 1(B)** and **(C)**).

3.2. Occurrence of Dinitrogen-Reductase Protein in *Aphanizomenon ovalisporum* Cultures

Protein extracts of culture samples (combined nitrogen grown and nitrogen fixing ones) and isolated heterocysts were subjected to SDS-polyacrylamide gel electrophoresis and Western blotted. The blots proved the presence of nitrogenase complex in heterocysts (**Figures 1(C)** and **2**) which was not detectable in extracts of combined nitrogen grown vegetative cells (**Figures 1(C)** and **2**). Increased amount of cell extracts of heterocysts loaded to polyacrylamide gel and transferred to nitrocellulose paper provided a linear signal increase for nitrogenase (**Figure 2**).

3.3. Alteration of Cylindrospermopsin Content of Combined Nitrogen and Nitrogen Fixing *Aphanizomenon ovalisporum* Cultures

We analyzed the alterations in cylindrospermopsin content of control and combined nitrogen starved (nitrogen fixing) cultures and isolated heterocysts with the help of capillary electrophoresis. When combined nitrogen grown *A. ovalisporum* cells were transferred from nitrogen efficient medium to dinitrogen, concomitant with the differentiation events the CYN content of the cells was decreased to a minimum value at 150 h (**Figure 3**). After this period of time the CYN content of cultures increased to about 95 % of control level (**Figure 3**). In control, combined nitrogen grown cultures the CYN content of *A. ovalisporum* cells was stable and the amount of CYN did not change on cell number base throughout the experiment (**Figure 3**). It is worth to mention that in nitrogen

fixing cultures CYN content of cells started to rise (150 h) when the increase of nitrogenase level was detected concomitantly (**Figures 1(C)** and **3**).

3.4. Amplification of PS Gene and PKS Gene in Vegetative Cells and Heterocysts of *A. ovalisporum*

The vegetative cells (as positive control) and heterocysts of the strains of *A. ovalisporum*, a well-known CYN producing strain (ILC-164) were tested for PKS and PS genes to investigate whether the gene rearrangement affects the detection of genes responsible for CYN biosynthesis. The amplified genes were detected in the vegetative cells and heterocysts too as shown on **Figure 4**.
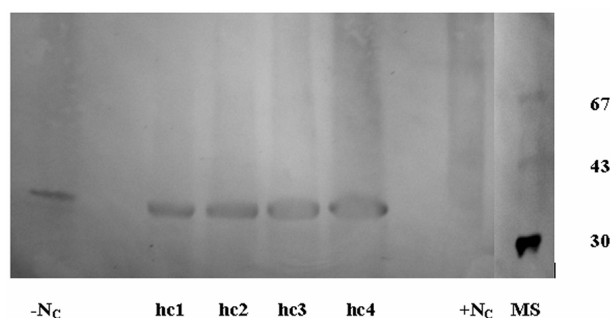
Figure 2. Presence of dinitrogenase reductase subunits in vegetative cells and heterocysts. Extracts of nitrogen fixing ($-N_c$, 25 μg protein) filaments and isolated heterocysts in increasing amount [hc1 (25 μg); hc2 (50 μg); hc3 (75 μg), hc4 (100 μg protein)] and combined nitrogen grown filaments ($+N_c$, 25 μg protein) were blotted and are shown by one representative experiment.
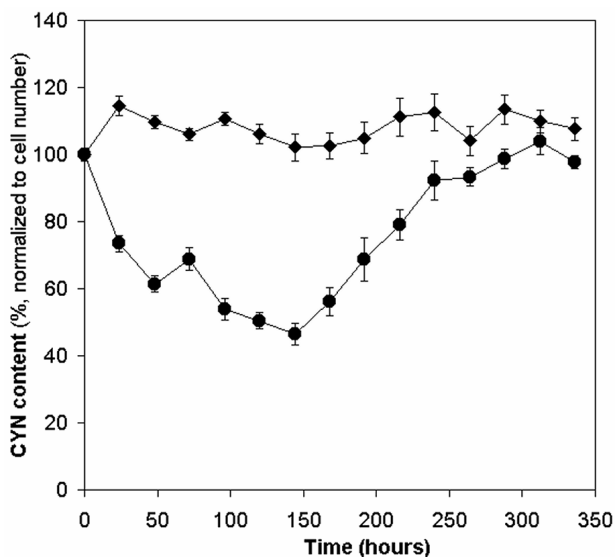
Figure 3. Alteration of cylindrospermopsin content of control (—◆—) and nitrogen fixing (—●—) cultures. In time 0 sample, the basal level of cylindrospermopsin was 1.91×10^{-4} ng per cell.
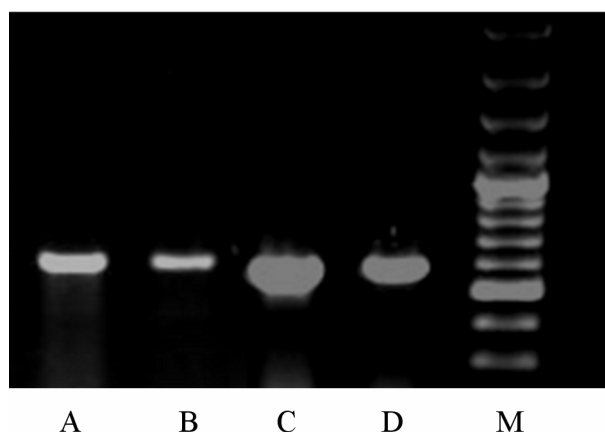
Figure 4. Presence of PKS and PS genes in vegetative cells and heterocyst. PKS in combined nitrogen grown filaments (A) and isolated heterocysts (B); PS in extracts of combined nitrogen grown (C) and isolated heterocysts (D); marker (M).

3.5. Cylindrospermopsin Content of Heterocysts

In order to ascertain whether the isolated heterocysts contain CYN, heterocysts of 10 l culture of *A. ovalisporum* (127 mg dry mass) were isolated and after cyanotoxin extraction the extracts were assayed for CYN by capillary electrophoresis (**Figure 5**). **Figure 5(A)** presents the capillary electrophoretic separation of compounds of isolated heterocysts and no CYN was detected. To show the unequivocal identity of CYN peak in the electropherogram the capillary electrophoretic run was spiked with HPLC-purified CYN standard (insert of **Figure 5(A)**). CYN was purified as our laboratory described earlier [27] and its electropherogram is shown on **Figure 5(C)**. In contrast to the isolated heterocysts, the cyanotoxin extracts of combined nitrogen grown cells contained CYN (**Figure 5(B)**).

4. Discussion

Filamentous cyanobacteria grown in presence of a source of assimilable combined nitrogen consist of undifferentiated vegetative cells. Following transfer to medium free of nitrogen source, many species, among those *Aphanizomenon ovalisporum* develop specialized cells known as heterocysts at regular intervals in each filament. Combined nitrogen depletion results in striking changes in the morphology, ultra structure, pigment composition, photosynthesis, nitrogen fixing activity, DNA structure etc. of nitrogen fixing cyanobacteria [1-6,20]. The heterocysts are sites of anaerobic nitrogen fixation and they are formed concomitantly with combined nitrogen depletion. The molecular basis of those processes is well studied and understood [2,3,5,6,29].

Toxic bloom developments of cyanobacteria caused mainly by eutrophication of surface water bodies, both in
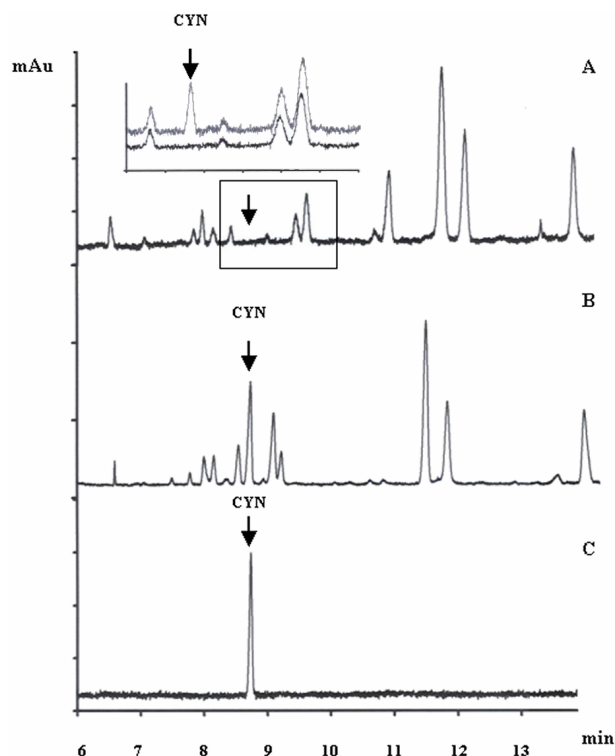

Figure 5. Capillary electrophoretic separation of CYN from heterocysts of *Aphanizomenon ovalisporum*. Insert in (A) shows a representative experiment which was spiked with 5 µg·ml⁻¹ CYN. CYN content of the combined nitrogen grown culture (B). Electropherogram of HPLC-purified CYN standard (10 µg·ml⁻¹).

marine and fresh waters are a worldwide problem. In addition toxic cyanobacterial blooms imply a high risk for human and animal health due to the ability of several species to produce potent toxins [7]. Cylindrospermopsin, a water soluble cyanotoxin is described as a potent heptotoxin with additional affection of kidneys, heart, thymus, spleen and intestine [8,12-14,30]. It is believed that the damage is chiefly caused by inhibition of protein synthesis [31-34]. What is more, carcinogenic and allergic activities were suggested recently due to the presence of uracil and sulphinated guanidino moieties [35-37]. In addition, [27] demonstrated that CYN interferes with plant metabolism as well. The biosynthesis of CYN is understood [26] and its chemical structure and feeding experiments with stable isotopes suggested the role of polyketide synthase [38]. [39] recommended that cyanobacterial amidinotransferases are likely to be involved in the formation of guanidino-acetic acid, a precursor of CYN.

Nutrient limitation stress and its regulation in cyanobacteria may have much common with heterotrophic bacterial nutrient restriction [40]. Therefore, one would anticipate the influence of nitrogen starvation on the metabolism of cyanotoxins, herein on CYN. It has been long

known, the combined nitrogen starvation results in a transient stop of growth of cyanobacteria capable for nitrogen fixation. Later, as consequence of alteration of gene expression, formation of heterocysts and nitrogenase, the growth resumes, though not necessarily at the same rate [6,25]. In this respect *A. ovalisporum* behaved as it was suspected under nitrogen shift down (**Figure 1**). Nevertheless, no data are available how nitrogen starvation alters the cyanotoxin content of CYN producing and nitrogen fixing *A. ovalisporum* cells. In addition, spatial and temporal variation of cyanotoxin content and toxicity of bloom samples is well documented [11,15,41-43]. In previous studies of microcystin production of organisms that do not fix nitrogen (e.g. *Microcystis aeruginosa*) it became clear that nitrate deprivation alters the cyanotoxin production. [42] demonstrated conclusively that microcystin content of cells and cell division is tightly coupled in nitrogen limited cultures. In the case of des-metyl-3-micro-cystin-RR producing *Oscillatoria agardhii* (not N_2 fixing cyanobacterium) high toxin production correlated with high combined nitrogen concentration in the culturing medium [44]. Basically similar data were obtained for microcystin producing *Anabaena* strains, but under nitrate grown conditions only [45]. Our finding suggest for a nitrogen fixing organism (*A. ovalisporum*) that the cyanotoxin content (CYN) of cells is dependent on metabolic activity of cells which most of the cases was correlated with growth rate (**Figures 1** and **3**). However, when nitrogen limitation started the CYN content of cells dramatically decreased and resumed only after an efficient nitrogenase functioning which supported the increase of growth rate of nitrogen fixing cells. In addition, our former study undoubtedly proved for the same organism (*A. ovalisporum*) that sulphur and phosphorus limitation changed CYN, a sulphur containing cyanotoxin production of cells in a similar manner [19].

It is known that isolated heterocysts are highly active in light driven nitrogen fixation measured by acetylene reduction and in protein synthesis [46-48]. Although the heterocysts contained the PKS-PS genes necessary for CYN biosynthesis (**Figures 3** and **4**), the isolated heterocysts of *A. ovalisporum* did not contain CYN as shown by capillary electrophoresis (**Figure 5**). The breakdown of reserve molecules during heterocyst formation and dramatic alteration of gene expression in heterocysts has been noted long ago [2,3,5,6,16,20,25, 49,50]. The current study indicates that the CYN content of isolated heterocysts was reduced in *A. ovalisporum* under combined nitrogen starvation. This is not surprising, since it is well known that during heterocyst differenttiation no net increase in cell mass of filament is possible and it is concomitant with basic alteration of heterocyst and vegetative cell metabolism [25]. Taking into account that the heterocyst focuses on nitrogen fixation under intra-

cellu-lar anaerobic conditions, it may be that in such circumstances, the toxin production is blocked. We suggest that these metabolic changes might be the reason of the disappearance of CYN during heterocyst differentiation.

5. Conclusion

Our findings suggest for the nitrogen fixing *A. ovalisporum* that the CYN content of cells is dependent on metabolic activity of cells, which is correlated with growth rate. CYN content of cells dramatically decreased when nitrogen limitation started and resumed only after an efficient nitrogenase functioning, which supported the increase of growth rate. The current study indicates that the CYN content of isolated heterocysts was reduced in *A. ovalisporum*, although the PKS and PS genes responsible for CYN biosinthesis were present in differentiated heterocysts.

6. Acknowledgements

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List of Abbreviations

CYN: cylindrospermopsin