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Dynamics of Cylindrospermopsin Production and Toxin Gene Expression in *Aphanizomenon ovalisporum*

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Abstract

***Aphanizomenon ovalisporum* is a cylindrospermopsin (CYN)-producing cyanobacteria species that due to its increasing worldwide distribution has become an important health alarm in the last few years. Several clusters of genes involved in CYN production have been described in different CYN$^+$ cyanobacteria genera, named *aoa* for *Aphanizomenon* and *cyr* for *Cylindrospermopsis* and others strains. The sequences of those genes are highly similar, but a rearrangement in gene order is also observed. The information on the control of CYN production by gene expression is still scarce, especially in *Aphanizomenon*. To obtain further information about the control of CYN production in *A. ovalisporum*, we have quantified the intra and extracellular CYN content, during nine days in BG11 batch cultures under optimal conditions. In parallel, the expression of four genes related to CYN synthesis, *aoaA-C* and *cyrJ*, has been analyzed by real time q-PCR. The results show a similar pattern of total CYN accumulation and gene expression. Most of the CYN is found intracellularly. Considering the high nitrogen content in the CYN molecule, we have explored if nitrogen assimilation could be related to CYN synthesis. We found inside the *aoaA* and *aoaC* sequences several putative binding domains for the global nitrogen regulator NtcA. The pattern of the *ntcA* expression along the culture is similar to that of CYN accumulation. Our data suggest that CYN production in *A. ovalisporum* seems to be controlled both by the expression of genes*aoa* and *ntcA*, this last one suggesting the influence of available nitrogen; however, other regulation mechanisms of CYN synthesis cannot be discarded.**

Keywords

Cyanobacteria, Cyanotoxins, *aoa* Genes, *cyr* Genes, *ntcA* Gene, Gene Regulation

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1. Introduction

Aphanizomenon ovalisporum is one of the toxic bloom-forming cyanobacteria in freshwater systems. It shows a potential invasive character, due to its high adaptability to different environmental factors [1]-[3]. It seems to be worldwide distributed, having been detected amongst other regions in Australia, Europe, Middle East and United States [4]. In the last decades, the presence of *A. ovalisporum* has become an important health hazard, because all strains found, with only one exception in Israel [5], produce the toxic alkaloid cylindrospermopsin (CYN).

Various cyanobacteria species have been identified as CYN producers. The gene clusters (*cyr* genes) involved in CYN synthesis have been completely described in *Cylindrospermopsis raciborskii* AWT205 [6] and CS-505 [7], *Aphanizomenon* sp. 10E6 [8], *Aphanizomenon* sp. 22D11 [8], *Oscillatoria* sp. PCC6506 [9] and *Raphidiopsiscurvata* CHAB1150 [10]. In the case of *A. ovalisporum* [11], only a partial description of the genes has been reported (*aoa* genes). All *cyr* and *aoa* clusters characterized are highly similar with respect to nucleotide sequences; however, they show several rearrangements in gene order and different flanking regions that might be involved in the expression of *cyr/aoa* genes. Such is the case of *hyp/hup* sequences founded in *C. raciborskii* AWT205 [6] [12], which could have NtcA binding sites, as they have been drecribed in *Nostoc* sp. PCC 73102 [13]. Bioinformatic analyses identified putative NtcA binding sites within the *cyr* gene cluster of *C. raciborskii* CS-505 and in Raphidiopsisbrookii [12] [14]. This fact and the high N content in the CYN molecule suggest the influence of N metabolism in the synthesis of the toxin. In general, nutrients seem to modulate CYN production, since nitrogen depletion [15] [16], phosphate and sulphate starvations cause significant changes in the toxin production [17].

The model of the CYN biosynthesis pathway [6] [18] includes the activity of an amidinotransferase (AMDT) in the first step, encoded by the *aoaA*/*cyrA* gene. The synthesis proceeds by the action of an enzyme complex constituted by a non-ribosomal peptide synthetase (NRPS)-polyketide synthase (PKS), codified by the *aoaB*/*cyrB* gene, and further by a PKS encoded by the *aoaC*/*cyrC* gene. Other PKS activities and tailoring genes are necessary to complete the synthesis, including the sulfotransferase codified by the *cyrJ* gene, which was proposed as a genetic marker to detect CYN-producing strains [5].

In *A. ovalisporum*, Shalev-Malul and coworkers [16] identified two transcriptional start points in the *aoaA* and *aoaC* genes, suggesting two modes of regulation of gene expression, one constitutive and the other alternative in response to environmental conditions, such as light intensity and nitrogen depletion [16], or inorganic phosphate (Pi) deprivation [19]. Moreover, a transcription factor, AbrB-like protein, has been proposed to regulate CYN synthesis, its binding region being located between *aoaA* and *aoaC* [16].

However, in *C. raciborskii* CS-505, transcriptomic analyses of four *cyr* genes (*cyrB*, *cyrI*, *cyrJ* and *cyrK*) under different nitrogen sources have shown almost no variation in gene expression, indicating only a constitutive expression [12]. But, different regulation points for individual genes have been observed.

Although diverse studies on CYN accumulation under different environmental conditions have been carried out, limited work has been focused in the relationship between gene expression and toxin production. In addition, the lack of standardization in experimental conditions makes it difficult to draw general conclusions on the possible gene regulation of CYN synthesis. The current work have the significant purpose to obtain further insights into the regulation of CYN production in *A. ovalisporum* we have performed BG_{11} batch cultures under optimal conditions, and analyzed the CYN content and the expression of the *aoaA*-*C cyrJ* and *ntcA* genes over 9 days. Additionally, NtcA putative binding sites inside the *aoa* cluster were searched, in order to link toxin production to nitrogen regulation.

2. Materials and Methods

2.1. Culture Conditions

Aphanizomenon ovalisporum UAM-MAO strain [20] was used throughout the work.

Three independent experiments were performed with batch cultures in BG_{11} [21], at 30˚C, under continuous white light (60 µmole photons $m^{-2} \cdot s^{-1}$) and bubbling with air passed through a 0.22-µm-pore-size filter. Culture samples were harvested every 24 h during 9 consecutive days. All plots were analyzed in triplicate. Cell observations were done with an Olympus BH-2 microscope at 400x magnification equipped with a Leica DC300F digital system.

2.2. Growth Parameters

Growth was followed both by biomass (optical density at 750 nm, $O.D._{750\ nm}$) and Chlorophyll *a* (Chl_a) content. Chl_a was extracted in 90% of methanol, and quantified as described by Marker *et al*. [22].

2.3. RNA Extraction and qPCR

RNA purification was performed using the RNeasy Mini Kit from Qiagen, following manufacturer instructions. Cells were collected from 5 mL of culture by filtration through 0.2 µm Nylon filters. The filters were washed twice with 50 mL of Milli-Q water, and frozen rapidly for RNA extraction. RNA samples were treated with DNAse to remove DNA, and DNA absence was evaluated by standard PCR reaction.

RNA quantification was performed by using a Nanodrop® *ND*-1000 spectrophotometer. RNA integrity and quality was determined by utilizing the Bioanalyzer 2100 (Agilent Technology, USA).

RNA samples were transcribed to cDNA utilizing randomprimers p(dN)9 and the high capacity RNA to cDNA Kit (Applied Biosystems).

Based on the genome of *A. ovalisporum* [11] PCR primers were designed to amplify three *aoa* genes. The sequence of *cyrJ* primers was designed from that of *Aphanizomenon*10E6 [8]. The *ntcA* primers were devised from *Nostoc* PCC7120 sequences [23]. The 16S *rRNA* gene was used as a reference, using primers designed from *A. ovalisporum* UAM289 [2] (**Table 1**). The right sequence of the expected amplicons was confirmed by sequencing previously to initiate the qPCR assays. Besides, similar amplification efficiency for target and the reference genes was ratified by carrying serial dilutions.

Real-time PCR was performed in 10 µL volume, including 5 µL Master Mix (SYBR Green, TOYOBO, Japan) and 0.25 µL of each primer (final concentration, 250 nM). The amplification reactions were performed in a AB7.900HTFast Real Time cycler (Applied Biosystems), under the following conditions: one cycle at 50˚C for 2 min, one cycle at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 s, 60˚C for 60 s, and 72˚C for 30 s. Each reaction was run in triplicate. The expression of 16S rRNA was stable under those specific conditions.

Gene expression data from the qPCR amplification were evaluated using the Ct values, and the 16S rRNA-gene was used as a control to normalize the expression levels of target genes. Relative transcription was calculated using the 2-ΔΔCt method, where $\Delta\Delta Ct = (Ct_{target} - Ct_{16S})_{time\ x} - (Ct_{target} - Ct_{16S})_{time\ 0}$, according to the handbook of Fast Real Time cycler-Applied Biosystems.

2.4. CYN Content Determination

CYN content was determined as previously described [20]. CYN standard was provided by Abraxis®.

2.5. Sequence Analysis and Data Representation

Putative NtcA binding domains in the *aoa* cluster (11.35 Kb) described by Shalev-Alon *et al*. [11] were searched

Table 1. qPCR primer sequences.

Gene	Function	qPCR Primer sequence (5'-3')	Amplicon size (nt)
16s *rRNA*	Ribosomal RNA	q16F CAGTAGCTGGTCTGAGAGGATG q16R GTAGGAGTCTGGGCCGTGT	62
aoaA	AMDT, 1st step CYN biosynthesis	qaoaAF ACCATTCTTTGAGGTAGAGAATCAA qaoaAR AATTTCGTTCCCAAAGGTGA	76
aoaB	NRPS/PKS, 2nd step CYN biosynthesis	qaoaBF TCTCCCCAATAATCTCGCTTAC qaoaBR TGTTCCATCAAGATCCCTTTG	75
aoaC	PKS, 3rd step CYN biosynthesis	qaoaCFGGCACCTGATGGTTGTTGTA qaoaCRCTTCGCCTCGCACATAGC	62
cyrJ	Sulfotransferase, CYN biosynthesis	qcyrJF ATTTCTTTGGGTTGGCGAAT qcyrJR AGACCATGGGAATTGAGTGG	67
ntcA	Transcriptional regulator in N metabolism	qntcAF CGAAACGTTTGAACGCAATA qntcAR AGTAGACTCGTTCGGCAGGA	62

using the CLC sequence viewer® (version 6.8.2). All data analyses were performed using GraphPad Prism® 5.

3. Results

3.1. Culture Growth

A. ovalisporum UAM-MAO was grown in BG_{11} batch cultures, under conditions previously considered optimal for this strain, temperature 30˚C [24] and light (60 µmol photons $m^{-2} \cdot s^{-1}$) [2]. The growth was checked during nine days both by the Chl_a content and the biomass ($O.D._{750}$) (**Figure 1**). The growth pattern after each parameter was different. Thus, the increment of chlorophyll was practically exponential from the start of the culture, and reached its late exponential phase around the sixth day. However, the biomass exhibited a lag phase, followed by an exponential phase; and late exponential phase was reached at the ninth day. Therefore, only the biomass showed a typical bacterial growth curve. No remarkable morphological changes, neither heterocyst formation, was observed in the filaments along the experiment.

3.2. CYN Production

CYN production was followed by quantifying both internal and external toxin per biomass unit; the pattern of both fractions being very different. However, the internal and total CYN content had the same tendency, showing three well differentiated phases (**Figure 2**): an increment from the beginning to the 4th day of the experiment, a

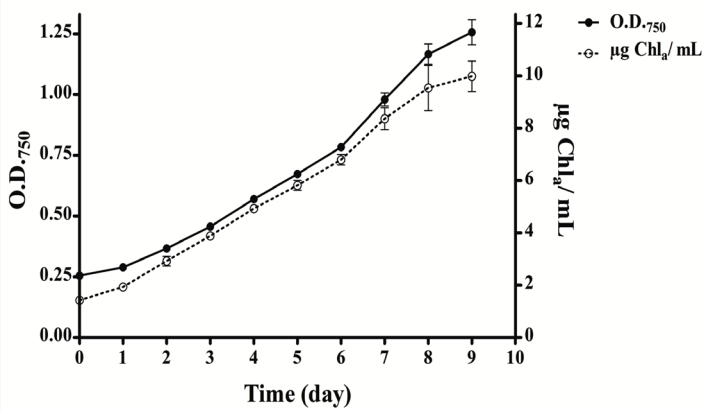

Figure 1. Growth of *Aphanizomenon ovalisporum* UAM-MAO expressed by biomass ($O.D._{750\ nm}$) and Chlorophyll a.

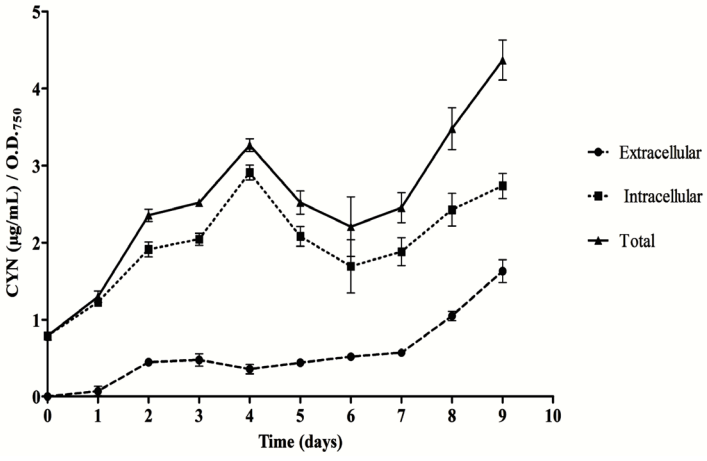

Figure 2. Cylindrospermopsin accumulation in *A. ovalisporum* UAM-MAO batch cultures. Intra-, extracellular and total CYN concentration (µg/mL) per biomass ($O.D._{750}$).

decrease in the following three days, and a new increment during the two last days. It is important to notice that the main contribution to total CYN content throughout the experiment was provided by the intracellular fraction being between 60% to 100%.

3.3. Gene Expression Analysis

The kinetics of *aoaA-C* gene expression was studied in parallel with that of CYN accumulation. The expression of the 3 genes showed a similar tendency along the experimental time period (**Figures 3(a)-(c)**): an increment between days 2 - 3, a lagduring days 4 - 5, and a further slow recovery in days 6 - 9, reaching the maximum levels during the last two days. The expression of *aoaA* was the earliestdetected, and the highest relative expression corresponded to *aoaB* and *aoaC*.

The kinetics of gene *cyrJ* expression was also compared with that of CYN accumulation. In *C. raciborskii*, *cyrJ* encodes a putative sulfotransferase involved in the lasts steps of the CYN biosynthesis pathway. Since the sequence of the homologous gene in *A. ovalisporum*is unknown, the sequence of *Aphanizomenon* 10E6 was used to design the primers for the expression analysis of *cyrJ*. The fluctuations of this genetranscript exhibited the same pattern of those of *aoa* genes, reaching the maximum levels on days 3, 8 and 9 (**Figure 3(d)**).

To study the possible influence of nitrogen metabolism in CYN formation, the expression of the *ntc*A gene was analyzed. The pattern of *ntcA* mRNA levels showed a similar trend to that of *aoaA-C* and *cyrJ* genes (**Figure 4**), the highest *ntcA* expression level being attained between the days 8 - 10.

By comparing the data of **Figure 2** and **Figure 3**, it is apparent that the kinetics of CYN production correlated with that of the relative expression of the genes involved in the CYN synthesis, *aoaA-C and CyrJ*, as well as with that of *ntcA*: the maximum toxin content between days 4 - 9 fitted with the highest gene expression.

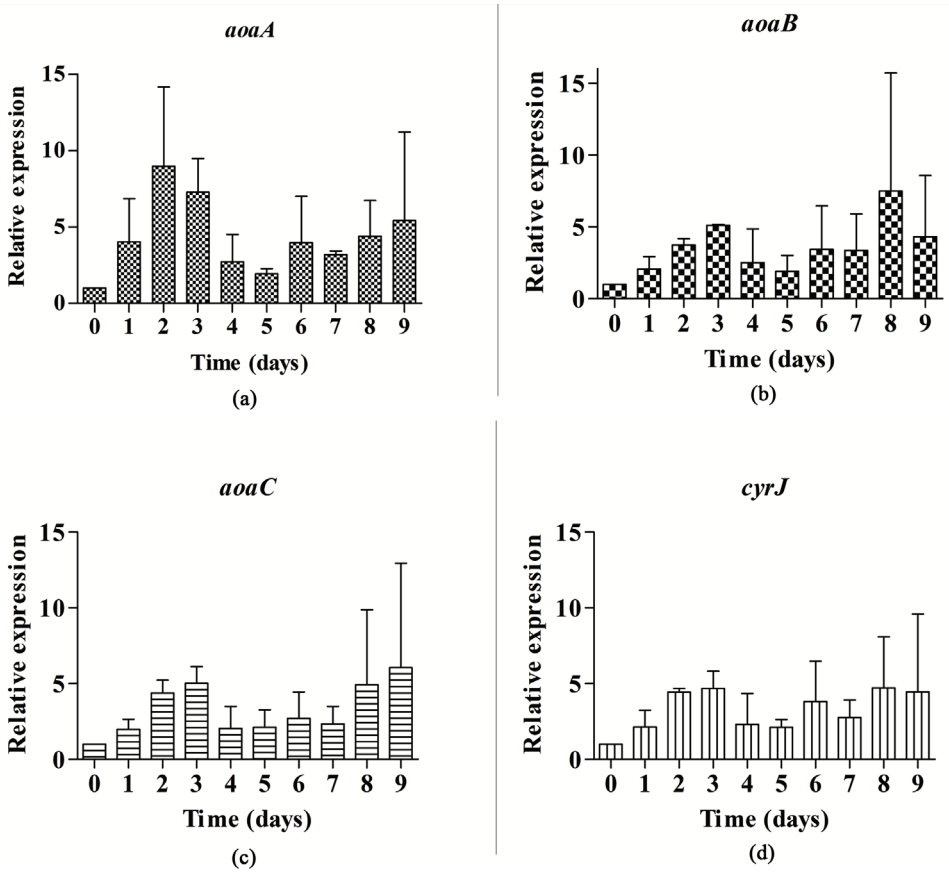

Figure 3. Expression of genes involved in CYN synthesis along a batch culture of *A. ovalisporum*. Relative expression values (normalized by 16S rRNA), RQ, are given. (a) *aoaA*, (b) *aoaB*, (c) *aoaC* and (d) *cyrJ* genes.

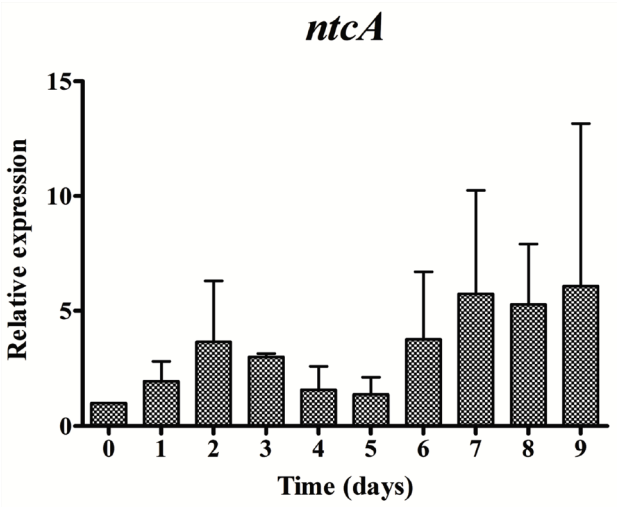

Figure 4. Expression of the *ntcA* gene along a batch culture of *A. ovalisporum*. Relative expression values (normalized by 16S rRNA), RQ, are given.

3.4. NtcA-Binding Regions

NtcA binding sequences, both canonical and non-canonical types, were searched along the *aoa* cluster (11.35 Kb) described for *A. ovalisporum* by [11]. In our search it was also taken into account other putative NtcAbinding motifs, as described by [25]. Three sequences were found inside the *aoa* coding regions (**Figure 5**): two were located inside the coding region of the *aoaC* gene, as GTA(N_7) TACN_{23}TAN_3T and GT(N_{10})ACN_{23}TAN_3T sequences; and the third one within the *aoaA* coding region, as GT(N_{10})ACN_{20}TAN_3T. The three sequences agree with those described −10 consensus-like box with the form TAN_3T, separated by 20 - 24 nt from the putative NtcA binding site upstream (**Figure 5**).

4. Discussion

The final aim of the present work was to contribute to the understanding of the regulation of CYN production in *A. ovalisporum*. We first analyzed the relationship between the CYN content and the expression of four genes considered to be involved in CYN synthesis, *aoaA-C* [11] [18] and *cyrJ*; and second, we explored if nitrogen metabolism could be related to the control of the CYN production, by comparing the toxin content and the expression of the nitrogen metabolism control gene *ntcA*, and searching for NtcA-binding sequences in the *aoa* cluster.

The experiments were carried out with batch cultures of the strain of *A. ovalisporum* UAM-MAO [26], grown with nitrate as N source and under other conditions described by other authors as optima for *A. ovalisporum*. The conditions used seemed appropriate, as indicated by the maximum growth rate attained, 0.24 day^{-1}, similar to the value range previously reported, 0.2 - 0.36 day^{-1} [2] [27]. The calculation of the growth rate was based on biomass increase, which under our conditions proved to be a more reliable criterion than chlorophyll. In effect, chlorophyll*a* variation along the culture did not permit to trace the expected growth pattern of a bacteria culture throughout the time of the experiment (**Figure 1**).

All analytical and gene expression measurements were carried out every 24 h along 9 days, just until late exponential phase of growth, to avoid nutrient depletion and the effects of possible inhibitory growth factors. To determine CYN production, both intra and extracellular contents of the toxin were quantified. Intracellular CYN was the main toxin fraction throughout the growth, representing 62.6% to 100% of the total toxin value (**Figure 2**). The greater cell CYN content is in accordance with previous studies with cultures of *A. ovalisporum* [2] [28] and with other data from our laboratory, obtained with another *A. ovalisporum* strain (VAC^+) and one of *C. raciborskii* (VCC^+) (data not shown), and disagrees with the general idea that in environmental samples CYN appears mainly dissolved in the surrounding medium [29] [30]. Nevertheless, an extracellular location, higher than 40%, could be expected under extreme environmental conditions, during the stationary phase of cyanobacteria

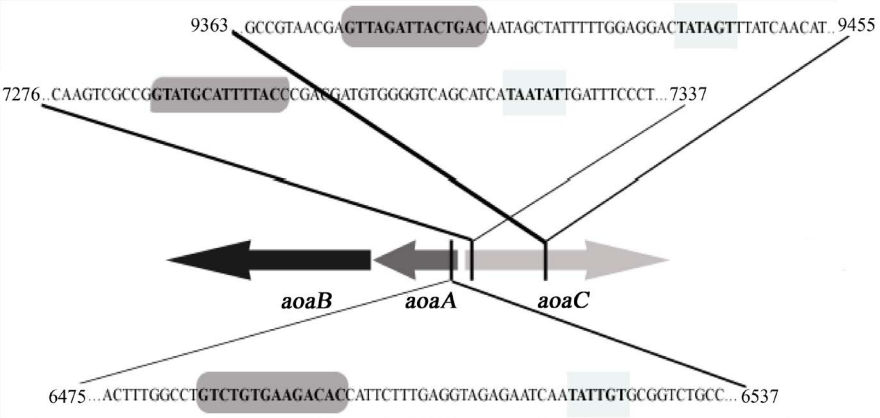

Figure 5. Putative NtcA boxes within the *aoa* cluster. Putative NtcA binding sites are represented in bold face and highlighted in grey. Regulatory conserved—10 box sequences (TAN_3T) are in bold face and enclosed in light grey rectangles. Location of putative NtcA sequences is marked by a thick black line within the cluster.

growth [31], and after cell lysis. The existence of CYN^+ strains from different species, *A. ovalisporum* included, in which the extracellular CYN was the major toxin fraction cannot be discarded.

The kinetics pattern of total CYN production correlates with that of *aoaA-C* and *cyrJ* (**Figure 3**) transcript accumulation, suggesting a control of CYN synthesis by those genes, at least under the conditions used. The fluctuations of both CYN and transcript levels were conspicuous at the early (days 3-4) and late exponential (days 8 - 9) phase. But in all instances, appreciable quantities of CYN and transcripts were observed, indicating a constitutive gene activity. This could justify a constitutive production of CYN in *A. ovalisporum*, as previously suggested [2] [17] [28]. Two transcriptional start points were described for *aoaA* and *aoaC* genes in *A. ovalisporum*, and differential expression levels of those genes were reported under diverse environmental conditions, suggesting the presence of two alternative promoters [16]. The distinct activity of each promoter might account for the fluctuations of both gene transcripts and CYN content.

CYN production, as well as the activity of *aoaA*-C and *cyrJ* genes, might be modulated by nitrogen metabolism, since the expression pattern of the N-master regulator gene *ntcA* (**Figure 4**) is similar to those of CYN content and the level *aoa*/*cyr* transcripts. Three potential NtcA targets with −10 like box consensus sequence TGT-$N_{9/10}$-ACA, suggested by Ramasubramanian *et al*. [32] were located inside the coding region of *aoaA* and *aoaC* genes (**Figure 5**). Similarly to those described in *C. raciborskii* CS-505 and *R. brookii* D9 by Stuken *et al*. [14]. The putative NtcA sequences are non-canonical, but that is not necessarily an impediment to recognize NtcA, since some other sequences have been suggested for NtcA recognition [25] [33], which differ from the NtcA sequence $GTAN_8TAC$ reported as the optimal for NtcA binding. As far as we know, only one canonical intragenic NtcA box has been described in cyanobacteria [34], moreover 1762 intragenic NtcA binding region have been found in the Anabaena PCC7120 genome, presenting the abundance of NtcA targets and function in cyanobacteria [35]. The modulation of CYN synthesis by nitrogen metabolism seems logical, considering the alkaloid nature of the toxin and, therefore the high demand for nitrogen availability. Nitrogen metabolism participation might not be restricted to NtcA; it could well be that a metabolite from nitrogen anabolism or catabolism is also involved in CYN synthesis, either in the *aoa* gene expression or/and in posttranscriptional steps. At any rate, further molecular experiments are needed to confirm or discard our suggestions on the modulation of CYN synthesis by either or both NtcA and a nitrogen metabolite.

5. Conclusion

Here we report the pattern of four toxin genes expression during the *Aphanizomenon ovalisporum* cells growth, in the presence of nitrogen; besides, the dynamics of CYN accumulation was determined in the same assay. The data have showed parallel fluctuations on toxin content and gene expression, suggesting a control of the CYN production by the regulation of gene expression. However, appreciable quantities of CYN and transcripts have been detected, indicating a constitutive gene activity. Moreover, the expression of *ntcA* gene detected, as

well as the identification of putative NtcA binding sites, in the *aoa* cluster, might reveal the influence of N metabolism in the *A. ovalisporum* CYN production.

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