Confirmation of the role of β-N-methylamino-L-alanine (BMAA) in nitrogen metabolism of cyanobacteria

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by

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The publication of this report emanates from a project entitled *Confirmation of the role of β-N-methylamino-L-alanine (BMAA) in nitrogen metabolism of cyanobacteria* (WRC Project No. K8/1076)

EXECUTIVE SUMMARY

BACKGROUND

β-*N*-methylamino-L-alanine (BMAA) is a neurotoxic non-protein amino acid produced by most cyanobacteria. Commercial ELISA kits have proven inaccurate. HPLC/MS analysis is expensive and requires a high level of expertise making it unsuitable for routine bulk water testing. Our recent observations that BMAA content of cyanobacteria is significantly controlled by medium nitrogen in culture might make it possible to produce a predictive model based on environmental parameters such as chlorophyll a content and phosphorus and nitrogen concentrations in water. However, in order to understand the dynamics of these modulating parameters so as to produce a robust model in future, the mechanism by which they function needs to be understood. A biochemical-mechanism approach is, however, overly complicated at this point, and a putative role for BMAA has been identified which allows for a simple confirmation of this role, and the acquisition of information required to produce a robust predictive model.

RATIONALE

Data emanating from WRC Research Project K5/2065 suggest a regulatory role for BMAA in nitrogen metabolism. Our recent work has confirmed an environmental association between environmental combined nitrogen and BMAA content of cyanobacteria, and the metabolic role of BMAA in the glutamate synthase – glutamine synthetase nitrogen assimilation pathway under ammonia deprivation. Confirmation of the role of BMAA in induction of nitrogen stress response at a transcriptional level would validate both the necessity for a long term monitoring project to develop a model for BMAA content based on easily measure physiochemical parameters, and, more importantly, it would provide the necessary biological basis for such a model, that will assure the confidence necessary to apply such a model to the protection of both water sector employees and the public in general.

Methodologically, monitoring of nitrogen status response gene transcription would allow for verification of BMAA as a nitrogen stress response regulator. This would provide a physiological basis for the gross environmental modulation of BMAA by nitrogen starvation and provide the necessary biochemical basis to hypothesize on the roles of phosphorous and light in the combined control of BMAA content in cyanobacterial blooms. This, in turn would offer a basis from which to minimize the environmental monitoring required to develop a model, but more importantly would ensure that the correct parameters were monitored for development and testing of the model.

OBJECTIVES AND AIMS

AIM 1

To determine whether BMAA initiates a transcriptional response similar to that induced by nitrogen starvation so as to understand the physiological basis for variation in the content of this neurotoxin in cyanobacterial blooms.

METHODOLOGY

The evaluation of transcriptional response of selected nitrogen starvation induced genes was done using quantitative Polymerase Chain Reaction (qPCR) methods. Genes were selected based on documented cyanobacterial responses to nitrogen starvation, which in turn was based on observed phenotypic changes to cyanobacteria on exposure to BMAA. The relevant genes selected were: *ntcA, glnB, glnN, nblA, rrn16S* (two sets). These were deemed sufficient to test the hypothesis that BMAA is a nitrogen starvation response regulator.

RESULTS AND DISCUSSION

BMAA did induce altered expression of transcripts of genes involved in the nitrogen starvation response. However, this increase was only significant for the global nitrogen regulator *ntcA,* and even in this case the increase was relatively small. The response of other genes involved in nitrogen assimilation was similarly small because the NtcA and PII proteins, at least in part, control them. Furthermore, although *NtcA* is a positive response regulator, its action is function depends on the presence of 2-oxoglutarate. Thus, although BMAA may indeed play a role in the induction of the nitrogen stress response, the full response does actually require nitrogen deprivation.

Aim 1

BMAA does appear to initiate a transcriptional response similar to that induced by nitrogen starvation, but the response does not result in observable physiological consequences as an immediate response to the BMAA under nitrogen replete conditions. This supports recently published environmental data that confirms nitrogen starvation induced production of BMAA by cyanobacteria.

GENERAL

BMAA is clearly involved in the nitrogen starvation response and development of a quiescent state, however the development of a full physiological response requires actual nitrogen deprivation and the concomitant relative increase in cellular carbon skeletons for nitrogen assimilation. Our recent discovery that Glutamate Synthase may use BMAA as a substrate

further suggests that in addition to the requirement for carbon skeletons, the transamination of the primary amino off BMAA may yield a keto-acid or cyclized form thereof that might be the more active compound. However, in the presence of ammonia and thus glutamine, this would not happen, which, we conclude, is why the BMAA induced response in the presence of combined nitrogen was limited.

CONCLUSIONS

BMAA is integral to the cyanobacterial nitrogen deprivation response. As such, prediction of combined nitrogen deprivation in a water body may be used to predict BMAA production in cyanobacterial blooms. The knowledge that BMAA does induce transcription of the global nitrogen response regulator *ntcA*, confirms the biological validity of the use of environmental combined nitrogen to predict BMAA production.

RECOMMENDATIONS FOR FUTURE RESEARCH

We strongly recommend a long term BMAA monitoring project on a major water source that does experience cyanobacterial blooms so as to develop and test a simple predictive model for BMAA based on environmental combined nitrogen fluctuations and cyanobacterial biomass.

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Dr S Downing	NMMU – Principal Investigator

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BMAA	β-*N*-methylamino-L-alanine
BOAA	β-*N*-oxalylamino-L-alanine
Bp	base pairs
cDNA	complementary DNA
C_T	threshold cycle
CYN	cylindrospermopsin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
glnB	encodes PII protein
*gln*N	encodes glutamine synthetase III (GSIII)
GOGAT	glutamine oxoglutarate amidotransferase
GS	glutamine synthetase
nbl	non-bleaching genes
NTC	non-template control
NtcA	transcriptional regulator in nitrogen metabolism
P_{II}	nitrogen-controlled signalling protein
PBS	phycobilisome
PCC	Pasteur Culture Collection
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	real-time PCR
T_M	melting temperature
*sig*C	gene encoding *s*igma factors σ^C

1 INTRODUCTION AND OBJECTIVES

β-*N*-methylamino-L-alanine (BMAA) is a neurotoxic non-protein amino acid produced by most cyanobacteria. Recent WRC projects (K5/1719 and K5/1885) have increased our knowledge on the analysis, environmental occurrence, biological origin, and the environmental fate of BMAA in freshwater systems. More recently, project K5/2065 has led to the discovery that the BMAA content of laboratory cultures of cyanobacteria is controlled predominantly by medium nitrogen, indicating the need for evaluation of this finding in a long term monitoring project aimed at the development of a predictive model. This is necessary because published methods for BMAA analysis are technically demanding, require sophisticated equipment, and are prohibitively expensive for the frequency of monitoring that should take place to limit public exposure to the toxin. The solution to this is the development of a predictive model to inform an alert level framework leading to analysis only when actually necessary. However, any models based on long term monitoring of physiochemical parameters will not be adequately robust if the relationship between the physiochemical parameters and the toxin has no biological relevance. In short, a model that predicts 'what' without reference to 'why' cannot be considered generally applicable.

Data emanating from K5/2065 suggest a regulatory role for BMAA in nitrogen metabolism. Confirmation of this would validate both the necessity for a long term monitoring project to develop a model for BMAA content based on easily measure physiochemical parameters, and, more importantly, it will provide the necessary biological basis for such a model that will assure the confidence necessary to apply such a model to ensure the protection of both water sector employees and the public in general.

1.1 BMAA AND NITROGEN METABOLISM

Recent studies (K5/2065) showed that the metabolism of BMAA in cyanobacteria is closely associated with cellular nitrogen status as depicted in Figure 1. The neurotoxic amino acid is produced under nitrogen-limited conditions and this negative correlation between cellular BMAA and combined nitrogen availability has been observed in both laboratory cyanobacterial cultures (Downing *et al.,* 2011) as well as in natural cyanobacterial blooms (Scott *et al*., 2014).

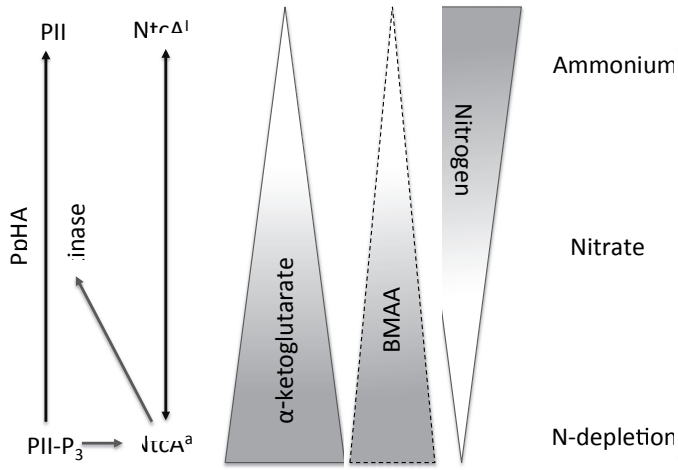

Figure 1: A schematic summary of the cellular responses that are controlled by the cellular nitrogen status. The global nitrogen response regulator NtcA and signal transduction protein PII are both inactive ($NtcA^I$, PII, respectively) in the presence of excess nitrogen and low α-ketoglutarate levels. Activated PII ($PII\text{-}P_3$) and activated NtcA ($NtcA_a$) function in a positive feedback loop stimulating the activation of each other under nitrogen deprivation. Dephosphorylation and inactivation of PII-P is catalysed by protein phosphatase (PpHA). BMAA is produced under nitrogen-limited conditions and disappears from the cell upon addition of ammonia. (Adapted from Schwarz and Forchhammer).

In addition, free cellular BMAA is rapidly removed following addition of combined nitrogen to nitrogen-depleted cyanobacterial cultures (Downing *et al.,* 2012). In the non-diazotrophic cyanobacterium *Synechocystis* PCC6803 the presence of exogenous BMAA results in the reversible induction of chlorosis and quiescence (Downing *et al.,* 2011), cellular responses that are characteristic to macronutrient acclimation responses including responses to cellular nitrogen deprivation. Although this strongly implicates BMAA in nitrogen metabolism, the role that this amino acid plays in nitrogen control in cyanobacteria is still unknown.

1.2 CELLULAR RESPONSES TO NITROGEN LIMITATION

Nitrogen metabolism is tightly regulated by the various interrelated regulatory elements that are in turn modulated by both physiological aspects such as growth phase and environmental factors such as nutrient availability. Most of these regulatory elements are proteins that function in the regulation of nitrogen controlled gene expression. However, ammonium and the keto acid, α-ketoglutarate (2-oxoglutarate) are key regulatory elements that function independently of gene expression.

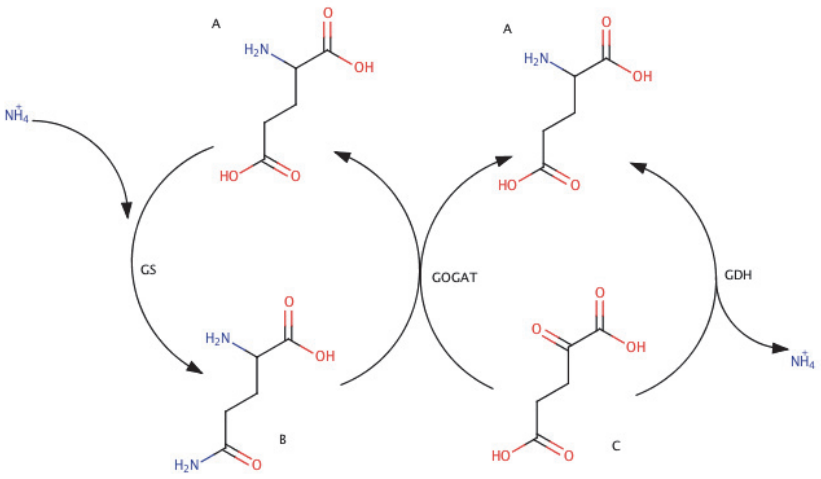

Figure 2: The glutamine synthetase-glutamine oxoglutarate amidotransferase (GS-GOGAT) pathway that is central to nitrogen assimilation in non-diazotrophic cyanobacteria. Nitrogen is assimilated in the form of ammonium (NH_4^+) via ammination of glutamate (A) to produce glutamine (B). Glutamate is produced via the transamination of α-ketoglutarate (C) by glutamine.

As illustrated in Figure 2, α-ketoglutarate is metabolized in the glutamine synthetase-glutamine oxoglutarate aminotransferase (GS-GOGAT) pathway, a pathway that is central in nitrogen metabolism in cyanobacteria (Flores and Herrero, 1994). Free cellular α-ketoglutarate is consumed during transamination with glutamine to produce glutamate and an accumulation of cellular α-ketoglutarate is therefore indicative of a decrease or absence in glutamine and ultimately a decrease or absence in ammonia (Muro-Pastor *et al.,* 2001; Sauer *et al.,* 1999). Due to its direct involvement in ammonium assimilation, the level of cellular α-ketoglutarate serves as a direct indication of the cellular nitrogen status and consequently functions directly in the activation of other nitrogen controlled response regulators or indirectly by modulating the expression of such regulators.

A recent study showed that in *Synechocystis* PCC6803 BMAA can act as a substrate for GOGAT (*pers. Comm.* Downing *et al*). This datum suggested that in *Synechocystis* BMAA is metabolized to yield a BMAA keto acid, however the regulatory role of this BMAA metabolite is still unknown.

In *Synechocystis* PCC6803, exogenous BMAA induces cellular responses that are characteristic of responses to nitrogen limitation. However, the amino acid induces such responses in the presence of nitrogen. This suggests that BMAA, or a BMAA metabolite,

may induce nutrient stress acclimation responses by acting specifically as a signal for nitrogen deprivation. Nitrogen starvation acclimation responses and the regulation of these responses are briefly discussed below.

1.3 THE NITROGEN DEPRIVATION RESPONSE GENES

NtcA, a DNA binding protein, is considered the central nitrogen metabolism response regulator in cyanobacteria (Schwarz and Forchhammer, 2005). It functions together with, and is activated by, PII, and its DNA binding and transcriptional activity is stimulated by α-ketoglutarate and repressed under excess nitrogen (Vazque-Bermudez *et al.,* 2003). NtcA activity is self-regulating with an increase in activated NtcA stimulating increased *ntc*A gene expression and a consequent substantial increase in the cellular levels of NtcA under nitrogen-limited conditions. Under nitrogen starvation NtcA and PII function in a positive feedback loop, where activated NtcA enhances the expression of *glnB* and PII phosphorylation via the activation of a protein kinase, while phosphorylated PII activates NtcA. Furthermore, NtcA dependent transcripts tend to increase as the concentration of CO_2 increases within the cell which may be due to either a direct increase in CO_2 or due to an increase in a CO_2 fixation metabolite (Garcia-Dominguez and Florencio, 1997).

*gln*B encodes GlnB, also referred to as protein PII, is a signal transduction protein that functions synergistically with the global nitrogen response regulator NtcA in the regulation of nitrogen controlled gene expression. Two promoter regions for the glnB gene have been identified in the in non-diazotrophic unicellular cyanobacterium, *Synechocystis* PCC6803, one of which is constituently expressed at low levels both in the absence of combined nitrogen as well as in the presence of ammonia and/or nitrate, while the second *gln*B promoter is only expressed under nitrogen deprivation (Garcia-Dominguez and Florencio, 1997). In *Synechocystis* PCC6803 various factors influence *gln*B expression. PII gene transcripts decrease when cells are transferred to the dark and in the presence of photosynthetic inhibitors, which suggest that expression of *gln*B is modulated by the cellular redox state (Garcia-Dominguez and Florencio, 1997). The nitrogen response regulator NtcA positively regulates the expression of glnB during nitrogen deprivation. PII is activated by phosphorylation that is positively controlled by the cooperative binding of ATP and α-ketoglutarate. At low levels or an absence of cellular ammonia, α-ketoglutarate levels are high and therefore PII protein is activated. In the presence of combined nitrogen, PII is dephosphorylated by a protein phosphatase rendering this regulatory protein inactive. PII protein expression and function is therefore closely associated with nitrogen metabolism and the regulation of nitrogen deprivation acclimation responses. Although the expression and activity of *gln*B are regulated by NtcA and α-ketoglutarate respectively, the existence of other

regulatory elements cannot be excluded (Garcia-Dominguez and Florencio, 1997). The effect of BMAA on the expression of this *gln*B is therefore of great interest.

Various sigma factors function in the regulation of gene expression specifically in response to nitrogen deprivation. Sigma factor SigC (σ^C) is a stationary phase specific regulator of *gln*B transcription under nitrogen-limited conditions (Asayama *et al.,* 2004). During stationary phase the cellular levels of this sigma factor is substantially lower compared to levels during exponential growth phase, with cellular σ^C levels remaining almost constant irrespective of cellular nitrogen depletion (Imamura *et al.*, 2003a; Imamura *et al.,* 2006). The specific mechanism behind the stationary phase specific activity of σ^C is not understood; whether σ^C is specifically activated during stationary phase, or whether all SigC RNA polymerase transcribed genes have additional regulatory elements, remains unknown. The expression of nitrogen controlled response regulators, PII and NtcA, is therefore influenced by the activity of SigC.

Chlorosis is a cellular state that is characterized by cell bleaching which is as a result of the degradation of phycobilisome (PBS) proteins (photosynthetic pigments) and the repression of PBS protein synthesis (reviewed in Schwarz and Forchhammer, 2005). The breakdown of thylakoid membranes, characteristic to quiescence, is accompanied by the degradation of photosynthetic apparatus and pigments, chlorophyll *a* and phycobilliproteins. During nutrient limiting conditions, such as nitrogen starvation, a metabolic balance needs to be maintained between the amount of energy produced and consumed. In order to maintain such a balance and reduce the cellular excitation rate when the rate of carbon fixation is reduced due to nutrient limitation, cells acclimate by reducing photosynthetic energy output *via* the degradation of PBS and as a result cells become bleached (Allen and Smith, 1969). During chlorosis cells maintain a very low metabolic activity, which ensures that, following the supply of the limiting nutrient, chlorosis can be reversed and cells can return to a normal vegetative state (Sauer *et al.,* 2001). The degradation of phycobiliproteins is tightly regulated and modulated by environmental triggers and the physiological and biochemical consequences of these triggers. The major regulatory elements that function in controlled pigment degradation during nutrient acclimation responses are the response regulators of the *nbl* pathway (reviewed in Schwarz and Forchhammer, 2005). Various components of this *nbl* pathway have been identified, however, the signal that triggers this pathway remains unknown, with preliminary data suggesting a role for changes in light and redox states (van Waasbergen *et al.,* 2002).

In *Synechocystis* PCC6803 exogenous BMAA induces a chlorotic response that is typical of cellular chlorosis observed in nitrogen limiting conditions. Like the chlorosis that occurs in response to macronutrient limitation, the chlorosis induced by BMAA is reversible, with complete recovery of pigmentation and growth of the culture. The link between BMAA and induction of chlorosis suggest the potential for a regulatory role of BMAA in pigment degradation.

In the cyanobacterium *Synechococcus* PCC7492 various components of the *nbl* pathway have been characterized. The polypeptide NblA, encoded by *nbl*A, (Collier and Grossman, 1994) is the main response regulator of the *nbl* pathway that regulates PBS degradation during nutrient limitation acclimation responses. Cellular NblA levels are upregulated under general nutrient starvation by other *nbl* regulatory components, NblR, NblS and NblC (reviewed in Schwarz and Forchhammer, 2005). NblR and NblS homologues have been identified in *Synechocystis* PCC6803 (Schwarz and Grossman, 1998; van Waasbergen *et al.,* 2002 and Frias *et al.,* 1993). In *Synechocystis* PCC6803, an NblA-like protein is required for pigment degradation during nitrogen limited conditions and is specifically expressed only during nitrogen starvation and not in response to sulfur limitation as in some other cyanobacterial species (Richaud *et al.,* 2001). *Synechocystis* PCC6803 contains two tandem *nbl*A gene copies, *nbl*A1 and *nbl*A2, both of which are transcribed from a single promoter and both of which are required for the synthesis of a functional NblA protein. These two NblA homologues function as a heterodimeric adaptor protein that mediates PBS degradation via a ATP-dependent protease (Baier *et al.,* 2014).

The expression of *nbl*A is unique in *Synechocystis* PCC6803 compared to other cyanobacterial strains in which *nbl*A expression is regulated by NblR (encoded by gene *nbl*R), which in turn is regulated by the sensor histidine kinase NblS (Zabulon *et al.,* 2007). In *Synechocystis* PCC6803 under nitrogen limitation *nbl*A expression and PBS degradation occurs even in the absence of nbl regulatory proteins NblR and NblS. Although PBS degradation a slightly delayed, the expression and functioning of NblA is unaffected in the absence of *dps*A (*nbl*S homologue) and *nbl*R-like genes. Therefore in *Synechocystis* PCC6803 the primary regulatory of PBS degradation is NblA, which is positively regulated by the global nitrogen regulator NtcA in the absence of combined nitrogen.

In *Synechocystis* PC6803 the regulation of photosynthetic pigment degradation that occurs during chlorosis in response to nitrogen starvation is therefore dependent on the interrelated regulation by the direct or indirect function of various response regulators including PII, NtcA,

α-ketoglutarate, NH_4^+. Therefore, the effect of BMAA on cellular pigments may involve the modulation by BMAA of any of the *nbl* pathway response regulator, *nbl*A or PII, NtcA, α-ketoglutarate or NH_4^+.

2 EXPERIMENTAL

Cyanobacterial cultures were exposed to BMAA in the presence and absence of combined nitrogen before the onset of natural nitrogen deprivation response, and the transcriptional response of selected genes m=determined.

3 EXPERIMENTAL PROCEDURES

An axenic *Synechocystis* PCC6803 culture was maintained in BG11 (Rippka, 1988) at a constant temperature of 21 ± 1°C under constant day:night cycle of 14:12 hours. Culture purity was confirmed microscopically during culture maintenance and prior to experimentation. An aliquot of this culture was sub-cultured over a period of three months, maintaining exponential growth and an excess of combined nitrogen in the culture medium by regular batch feeding. The affect of BMAA on gene expression was analysed during exponential growth phase.

Replicate volumes (30 mL) of a *Synechocystis* PCC6803 culture in exponential growth phase (culture density of OD_{750nm} = ~ 0.400) were aliquoted into 24 replicate culture tubes (Falcon™ 50 mL conical centrifuge tubes, Fischer Scientific®). Cells from these 24 replicate culture aliquots were harvested by centrifugation at 5000 x *g* for 10 min after which cell pellets from 12 replicate cultures were individually resuspended in BG11 media and the remaining 12 replicate cell pellets were individually resuspended in $BG11_{00}$ ($BG11_{00}$ is a modified form of $BG11_0$ media described by Rippka, 1988, in which all sources of combined nitrogen has been removed, see Downing et al., 2011). Six replicate aliquots resuspended in BG11 and $BG11_{00}$ (12 in total) were inoculated with BMAA (30 µL) at a final concentration of 100 µM. The remaining 12 control aliquots were inoculated with BMAA diluent, 20 mM HCl. All tubes were incubated at 21 ± 1°C at constant elimination at 17 umol.E^{-2}.s^{-1}. Three replicates of each culture regime (BG11, BG11+BMAA, $BG11_{00}$, and $BG11_{00}$+BMAA) were harvested at six and twelve hours following BMAA inoculation. Cell pellets were harvested by centrifugation as before and cell pellets were immediately resuspended in 1 mL TRIzol® reagent, transferred to 2 mL ribonuclease-free screw-cap homogenization tubes (Sarstedt®, Germany) and stored at -80°C until further processing.

RNA isolation and purification was preformed as follows: Cell pellets, re-suspended in TRIzol® reagent, were homogenized with a stainless steel homogenization bead using a Retsch™ M301 bead homogenizer, at a frequency of 30 (1 s^{-1}) for 5 min twice in succession. RNA isolation from homogenized cell samples was performed according the TRIzol® reagent (Ambion®, Life Technologies™) protocol with minor modifications. RNA isolation optimization showed that removal of particulate matter prior to phase separation with chloroform increased the quality and yield of RNA. Therefore, following homogenization, tubes were centrifuged at 14 200 x *g* for 10 min and the supernatant (free of particulate matter) was transferred to a sterile nuclease-free tube. Chlorophorm (0.2 mL) was added to the supernatant and mixed by repeated gentle inversion of the tubes after which samples were inoculated at room temperature for 2-3 min. Phase separation of the chloroform/ TRIzol® suspension was achieved by centrifugation at 12 000 x *g* for 15 min. The clear aqueous phase was removed, the volume determined to the nearest 100 µL, and transferred to a clean nuclease-free tube to which isopropanol (100%) was added in a 1:1 ratio (isopropanol:aqueous phase). Samples were mixed by gentle inversion and incubated for one hour at room temperature for 10 min. Following incubation, tubes were centrifuged at 14 000 x *g* for 15 min at 4°C, the supernatant discarded and the pellet washed in ice-cold 75% ethanol. Ethanol suspensions were centrifuged at 7500 x *g* for 5 min at 4°C and the supernatant discarded. Pellets were air dried at room temperature for 10-15 min. Dried RNA pellets were re-suspended in nuclease-free water (40 µL) and incubated at 55-60°C for 10 min to ensure complete resuspension of RNA. Immediately after RNA isolation and resuspension in water, RNA isolations were treated with RNase-free DNase I (Thermo Scientific) according to the manufacturers protocol with one minor modification.

As optimization reactions showed that deactivation of DNase I through the addition of EDTA and incubation at 65°C as specified in the manufacturer's protocol, may reduced the final yield and quality of RNA, this step was omitted. In stead, immediately after 30 min incubation at 37°C in the presence of DNase I (1U.μL^{-1}) RNA samples were purified from contaminating enzymes, proteins, deoxyribonucleotides and undigested DNA using an RNeasy® MinElute® Cleanup kit (Qiagen®). Quality and quantity of purified RNA samples were determined using an Agilent® RNA 6000 Nano assay on an Agilent® 2100 Bioanalyzer (Figure 3), which showed an average RNA concentration of ~80 ng.μL^{-1}. Purified RNA samples were immediately used in the synthesis of complementary DNA (cDNA).

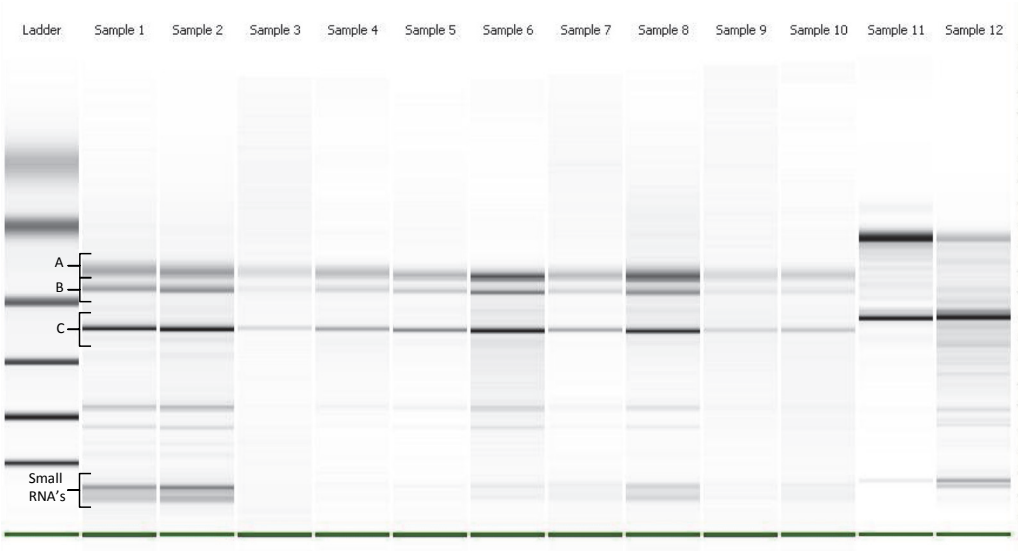

Figure 3: Agilent® 2100 Bioanalyzer generated gel showing the quality of selected RNA samples isolated from *Synechocystis* PCC6803. RNA integrity is determined based on (A) 50S rRNA 23S subunit, 2906 nucleotides, (B) 30S rRNA 16S, 1542 nucleotides and (C) 50S rRNA 5S subunit, 120 nucleotided.

Complementary DNA was synthesized from purified RNA using BioRad® iScript™ Reverse Transcription (RT) Supermix for RT-qPCR (Biorad®). The reaction mixture was scaled up to contain 10 µL purified RNA (0.8 µg RNA) in water, 8 µL iScript™ RT Supermix and 2 µL nuclease-free water in a final volume of 40 µL. Samples were incubated at 25 ˚C for 5 min (priming), 37 ˚C for 30 min (reverse transcription) and 85 ˚C for 5 min (enzyme inactivation) using a Biorad® MJ mini Personal Thermal Cycler. In order to evaluate the purity of RNA used in the cDNA reactions and the confirm that the RNA samples were free of contaminating genomic DNA, no-reverse transcriptase (no-RT) controls were included for each sample set. No-RT reactions were prepared according to the BioRad® iScript™ protocol using the no-RT-qPCR Supermix supplied. cDNA and no-RT controls was stored at -20°C until further use.

Gene transcripts of selected genes (transcription factor, *ntc*A; PBS degradation *nbl* pathway gene, *nbl*A1; signal transducting protein PII, *gln*B; and NtcA-controlled glutamine synthetase gene, *gln*N) were quantified by means of real time-PCR (RT-PCR) analysis using SsoFast™ EvaGreen® Supermix according to the manufacturer's instructions. 16*S rrna* was amplified as a reference gene and all primer sequences are shown in Table 1.

To account for instrument variations, all PCR reactions were performed in duplicate. Duplicate non-template control (NTC) reactions, a reaction that did not contain cDNA, were included for each gene primer set. RT-PCR reactions (20 µL) were prepared on ice and individual reactions were performed for a specific gene (one primer set per reaction). Reactions contained the following; SsoFast™ EvaGreen® Supermix (10 µL), forward and

reverse primer (1 µL each at a final concentration of 500 nM), total template cDNA (1 µg) and RT-PCR-grade RNase/DNase-free H_2O). The RT-PCR protocol was as follows: 1 cycle of 95°C for 3 min; 40 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s; 95°C for 30 s. A melting profile was determined by raising the temperature by 1°C every 30 s from 55°C to 95°C.

Table 1: Information on the RT-qPCR primers for selected genes

Gene		Primer sequences	Reference	Annealing temperature (°C)
*gln*B	F	5' -AGTAGAAGCGATTATTCGC-3'	Asayama *et al.*, 2004	55
	R	5' -TACCAACTTGTCAACAACC-3'		
*ntc*A	F	5'-CGGTGGGCTAGGGTTTCAAT -3'	NCBI	55
	R	5'-CACCGGGATATGGGTTCTCG -3'		
*gln*N	F	5'- CCTGGAAGATATGTGGGCTG -3'	Imamura *et al.*, 2006	61
	R	5'-CTGGATCCACCCAAATTTTCGTAATAGTCC-3'		
*nbl*A1	F	5'-ACCTAGGGGCTCCAGGGGAGCC -3'	Richaud *et al.*, 2001	55
	R	5'-TTGGAGGGGCAACAGCTATGAA -3'		
*rrn16*S	F	5'-CTGAAGATGGGCTCGCGT-3'	Imamura *et al.*, 2006	61
	R	5'-CGTATTACCGCGGCTGCT-3'		
*rrn16*S	F	5'-CACACTGGGACTGAGACAC-3'	Pinto *et al.*, 2012	61
	R	5'-CTGCTGGCACGGAGTTAG-3'		

The efficiencies of RT-PCR reactions were analysed using the $\Delta\Delta C_T$ method of relative quantification, as described in Critical Factors for Successful Real-Time PCR (Qiagen®). In this method the threshold cycle (C_T) values of the experimental control samples (calibrator) are directly compared to that of the experimental samples (sample) based on differences between each sample's C_T values for the target gene (i.e. an affected gene) and the reference gene (i.e. an unaffected housekeeping gene). The method calculates the ΔC_T value for each sample from which the $\Delta\Delta C_T$ value can be calculated. Equations are given below.

ΔC_T *(sample)* $= C_T$ *(target gene)* $- C_T$ *(reference gene)*

ΔC_T *(calibrator)* $= C_T$ *(target gene)* $- C_T$ *(reference gene)*

$\Delta\Delta C_T$ *(sample)* $= \Delta C_T$ *(sample)* $- \Delta C_T$ *(calibrator)*

Given comparable RT-PCR efficiencies of both the target gene and the reference gene the normalized level of target gene expression was calculated as follows:
normalized target gene expression level in sample = $2^{-\Delta\Delta_T}$.

Cells resuspended in nitrogen rich BG11 media (see section 4.2.1) were used as experimental control samples (calibrator) where experimental samples were cells resuspended in BG11 + 100 µM BMAA, nitrogen-free $BG11_{00}$, and $BG11_{00}$ + 100 µM BMAA. Data shown in section 5 therefore represent the gene expression levels in experimental sample cells relative to that of control samples.

4 RESULTS, TREATMENT OF RESULTS AND DISCUSSION

In order to account for variability in qPCR reactions as a result of variations in technical and experimental parameters such as RNA or cDNA quality and qPCR efficiency, qPCR results are normalized to a specific reference gene. Expression of reference genes should be constant irrespective of variations in growth parameters such as light:dark cycles, temperature or nutrient limitations. The 16S ribosomal RNA gene, *rrn16s*, was selected as reference gene for this study as its expression in *Synechocystis* PCC6803 is not affected by growth phase and is stable under both nitrogen limited and replete conditions (Pinto et al., 2012).

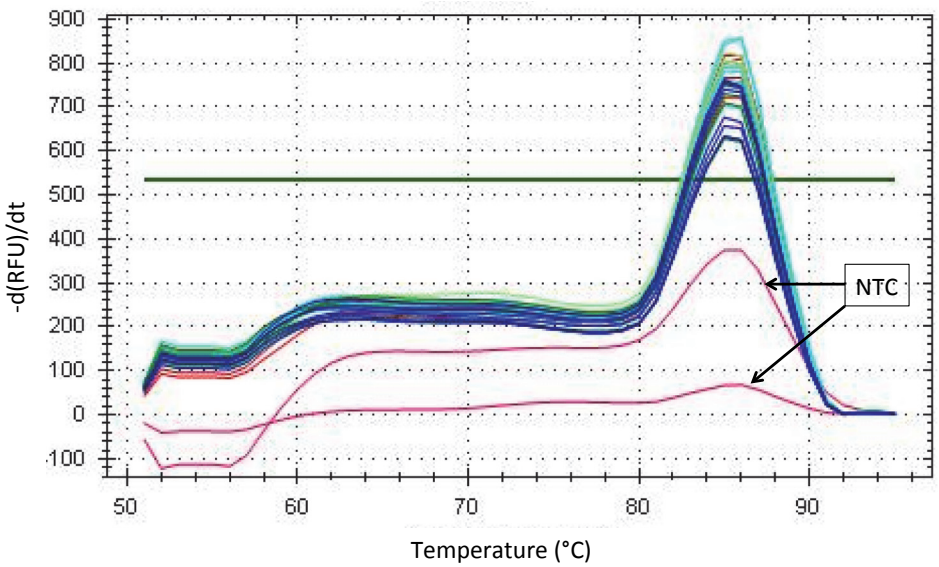

Figure 4: Melting curves of qPCR of the *rrn16s* gene reversed transcribed from RNA isolated from *Synechocystis* PCC6803 grown in the absence and presence of BMAA under difference nitrogen regimes. All experimental amplicons produced a single peak, and no template controls (NTC) traces remained below the threshold cycle (green) indicating no primer dimer formation.

Melting curve analysis of *rrn16s* amplicons, produced from cDNA synthesized from RNA isolated from *Synechocystis* PCC6803 grown in the presence and absence of BMAA under different nitrogen regimes, using primers 5'-CTGAAGATGGGCTCGCGT-3' (plus) and 5'-CGTATTACCGCGGCTGCT-3' (minus) (Imamura et al., 2006) confirmed the synthesis of a single unique *rrn16s* amplicon per PCR reaction, evidenced by single peaks during melt curve analysis of all experimental amplicons produced (Figure 4), with an amplicon melting temperature (Tm) of ~85˚C, and Cq median of ~16 and a NTC Cq of ~37. This verifies that this primer set used in the amplification of *rrn16s* did not produce any unwanted amplicons nor formed primer dimers.

Melting curve analysis of all selected gene amplicons produced from cDNA synthesized from RNA isolated from *Synechocystis* PCC6803 grown in the presence and absence of BMAA in both N- and N+, using respective primer sets (Table 1) confirmed the synthesis of a single unique amplicon per PCR reaction for each gene, evidenced by single peaks during melt curve analysis of all experimental amplicons produced. This verifies that all primer sets used in the amplification of selected genes did not produce any unwanted amplicons nor formed primer dimers.

The quality of cDNA used in the qPCR analysis of selected gene expression levels was confirmed by non-reverse transcriptase (No-RT) controls as specified by the BioRad® iScript™ Reverse Transcription (RT) Supermix for RT-qPCR. In short, RNA template was added to a No-RT reaction mixture, which contains the identical components as the RT reaction mixture, except it does not contain reverse transcriptase enzyme. qPCR analysis of No-RT controls therefore reveals the presence of contaminating genomic DNA. qPCR analysis of no-RT controls of experimental samples showed no amplification that was significantly greater than the no template controls (NTC). This confirmed that the cDNA samples were pure and free from significant amounts of contaminating genomic DNA.

The normalized expression levels of *gln*B and *ntc*A in *Synechocystis* PCC6803 grown in the presence and absence of BMAA in both N- and N+ growth media are shown in Figure 5.

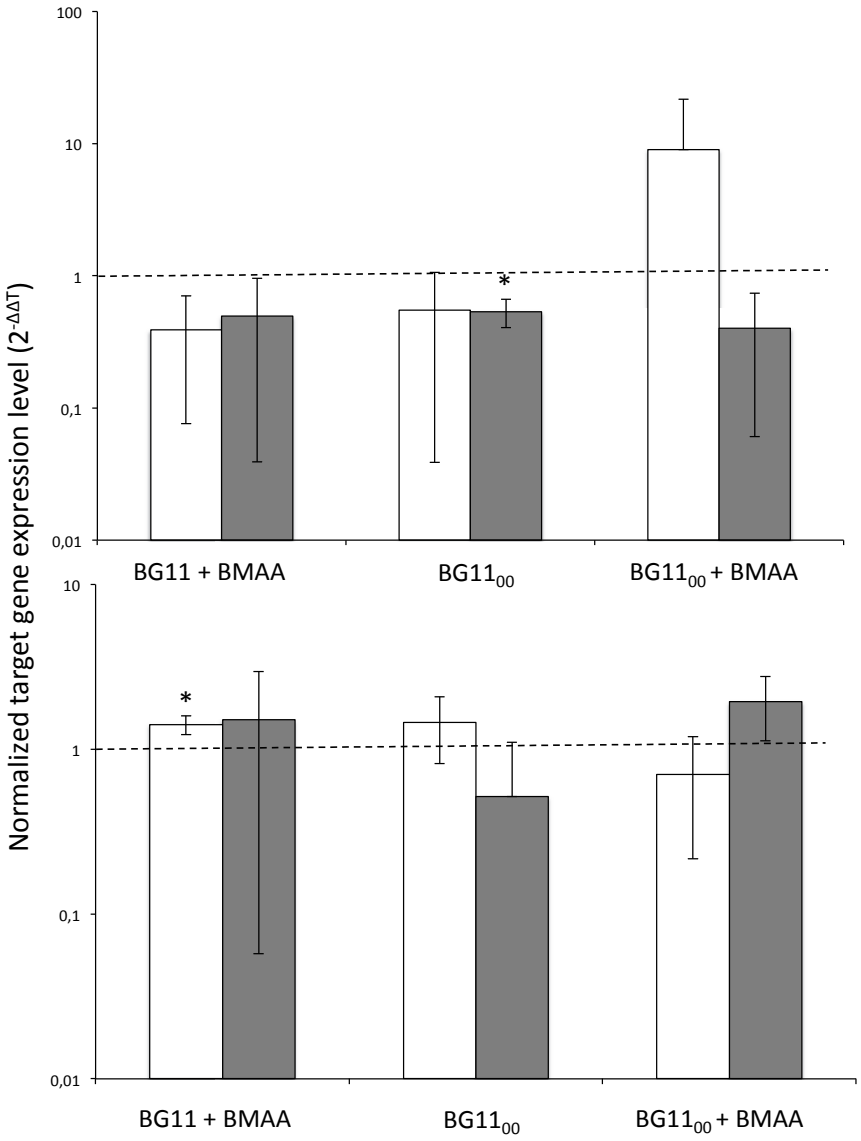

Figure 5: Gene expression levels (expressed as $\Delta\Delta C_T$) of nitrogen-controlled signal transduction protein, PII (*gln*B) (Upper pane) and the global nitrogen regulator NtcA (*ntc*A) (Lower pane), normalized to the reference gene *16S rrna,* in *Synechocystis* PCC6803 grown in the presence and absence of BMAA (100 µM) in both N- and N+ growth medium for 6 hours (open bars) and 12 hours (grey bars). Target gene expression levels in samples were calibrated against that of cells grown in N+ (BG11) medium in the absence of BMAA (dotted line). Error bars denote standard deviations where n=3. Significant (p<0.05) difference in gene expression levels from control cultures is indicated by (*).

GlnB (PII) functions, together with the global nitrogen response regulator NtcA, in the regulation of nitrogen controlled gene expression. Under nitrogen deprivation, expression of *gln*B increases as NtcA positively regulates it at a transcriptional level. In a study by Garcia-Dominguez and Florencio (1997) in which the expression of *gln*B under different nitrogen regimes were investigated, *gln*B expression levels increased rapidly once combined nitrogen was removed from the growth medium and reached a maximum expression level after 5 hours of nitrogen deprivation after which transcript levels remained high for the 5 hours to

follow. In the current study, *gln*B expression decreased relative to control cells grown in nitrogen-replete BG11 media in almost all treatments within 6 hours and did not increase over 12 hours. A relative increase in *gln*B expression was only observed in nitrogen-depleted $BG11_{00}$-grown cells in the presence of BMAA, however this increase was not significant. These data are not in accordance to other published reports on the regulation of PII expression in *Synechocystis* PCC6803 under nitrogen limiting conditions, under which an increase in the expression of this nitrogen controlled gene has been observed within a few hours of nitrogen step-down. Two promoter regions for the *gln*B gene have been identified in *Synechocystis* PCC6803, one of which is constituently expressed at low levels both in the absence of combined nitrogen as well as in the presence of ammonia and/or nitrate, while the second *gln*B promoter, containing a regulatory site, is only expressed under nitrogen deprivation (Garcia-Dominguez and Florencio, 1997). Considering that *gln*B is both constitutively expressed and expressed in response to nitrogen deprivation, data would suggest that the *gln*B gene expression quantified is this study was that of the constitutively expressed *gln*B promoter. Furthermore, this would suggest that the cells did not experience true nitrogen starvation within the experimental time period. However, the major regulator of *gln*B expression is NtcA, which functions together with sigma factors σD and σB (Asayama *et al.,* 2004) to up-regulate the transcription of this gene in the absence of combined nitrogen. Under nitrogen limitation, NtcA activity and the consequent transcription of *gln*B are also positively regulated by increased α-ketogluterate levels, themselves a function of nitrogen deficiency. The only significant increase in *ntc*A transcription levels relative to that in control samples was observed in cells grown in nitrogen rich BG11 supplemented with 100 μM BMAA. No significant increase in *ntc*A transcript levels were observed in cells grown in the absence of nitrogen as a control. These data would again suggest that cultures were not adequately nitrogen starved within the experimental time frame to induce the characteristic increased expression of both nitrogen controlled genes *ntc*A and *gln*B. Interestingly, the significant increase in *ntc*A expression observed in BG11 + BMAA cultures suggests that the presence of BMAA induces a cellular state characteristic to nitrogen starvation, but that the generalized response requires additional features of nitrogen starvation such as α-ketogluterate accumulation.

NtcA was the only significant increase in gene expression levels in cells exposed to BMAA relative to control cells. It can thus be concluded that although BMAA may induce *ntcA* expression, a cellular state of nitrogen depravation must also exist for additional genes to be transcribed. Factors, which include availability of NADPH reducing power and electron transport (Garcia-Dominguez and Florencio, 1997), cellular redox states and light (Alfonso *et al.,* 2001) and α-ketoglutarate (Flores and Herrero, 1994) also influence the expression of

*gln*B and the *nbl* genes. Based on the data and the timeframe of this study it would appear that the effect of BMAA on nitrogen deprivation-controlled processes such as PBS degradation is not controlled by BMAA directly at transcription level, but is rather a function of downstream cellular phenomena that requires a longer time to induce an effect on the transcription of applicable genes. Nonetheless, that *ntcA* is directly induced by BMAA under nitrogen replete conditions strongly supports a nitrogen response function for BMAA and validates long term environmental monitoring for predictive model development. However, environmental combined nitrogen status will no be sufficient for the development of a model. This is an important outcome of this research.

Two glutamine synthetase proteins, GSI and GSIII encoded by genes *gln*A and *gln*N, respectively are present in *Synechocystis* PCC6803. Both genes are under nitrogen controlled transcriptional regulation. GSI is constitutively expressed and account for 90% of glutamine synthetase activity under normal nitrogen replete conditions while GSIII only account for 3% (Reyes *et al.,* 1997). However, under nitrogen starvation conditions, the expression of *gln*N and the activity of GSIII rapidly increases so that GSIII activity accounts for up to 20 % of all GS activity within 24 hours of nitrogen step-down (Reyes *et al.,* 1997).

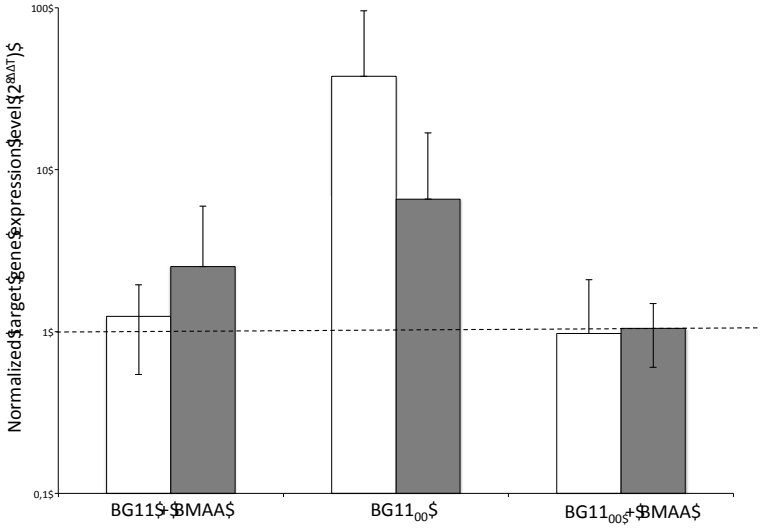

Figure 6: Gene expression levels (expressed as $\Delta\Delta C_T$) of nitrogen-controlled glutamine synthetase, GSII (*gln*N) normalized to the reference gene *16S rrna,* in *Synechocystis* PCC6803 grown in the presence and absence of BMAA (100 µM) in both N- and N+ growth medium for 6 hours (open bars) and 12 hours (grey bars). Target gene expression levels in samples were calibrated against that of cells grown in N+ (BG11) medium in the absence of BMAA (dotted line). Error bars denote standard deviations where *n*=3. Significant (p<0.05) difference in gene expression levels from control cultures is indicated by (*).

Although the exact mechanism is not well understood, gln*N* transcription during nitrogen starvation is positively regulated by NtcA, as *gln*N is not transcribed in mutant cells lacking *ntc*A. Nitrogen step-down and/or and increase in *ntc*A expression or NtcA protein levels

would result in a concomitant increase in the *gln*N expression. Figure 6 shows the *gln*N expression levels in the various cellular treatments over a total of 12 hours. As would be expected, a marked, but not significant, increase in *gln*N expression was observed within 6 hours in cells grown in the absence of nitrogen ($BG11_{00}$). No significant increase in *gln*N expression relative to control cells was seen in any of the treatment. Again, it appears that BMAA does not have a significant effect on GSIII at a transcriptional level.

In *Synechocystis* PCC6803 PBS degradation is primarily regulated, indirectly by the global nitrogen response regulator NtcA, and directly by die sensor kinase, NblA. In *SYnehcocystis* PCC6803 tandem *nbl*A genes are transcribed from a single promoter and are necessary for the synthesis of a functional NblA protein. Figure 6 shows the effect of BMAA on the expression on *nbl*A1 in the presence and absence of nitrogen. In *Synechocystic* PCC6803 NblA protein expression is increased within hours of nitrogen step-down and *nbl*A1 expression is fully induced within 4 hours (Richaud *et al*., 2001). Data here show that *nbl*A1 expression was significantly (p>0.05) enhanced following 6 hours of nitrogen step-down with ~5 fold increased expression relative to cells grown in the presence of nitrogen (BG11). *nbl*A1 gene expression remained elevated after 12 hours. This data is in accordance with what would be expected for *nbl*A expression under nitrogen limiting conditions (Richaud *et al.,* 2001; Baier *et al.,* 2014). The presence of BMAA in nitrogen deplete growth medium ($BG11_{00}$) did not seem to have a significant effect on *nbl*A1 expression. Interestingly, although not significant, *nbl*A expression, relative to cells grown in nitrogen replete media (BG11), was slightly elevated in cells grown BG11 in the presence of BMAA 6 hours after the addition of BMAA and even more elevated following 12 hours in the presence of BMAA. This may suggest that in *Synechocystis* PCC6803 BMAA affects the expression of *nbl*A1 even in the presence of nitrogen. In *Synechocystis* PCC6803 exogenous BMAA at a concentration ~20 fold less than that used in this study resulted in a significant increase in chlorotic cells within 24 hours of BMAA exposure, this in the presence of combined nitrogen (Downing *et al.,* 2012). Evidently under normal growth conditions exogenous BMAA has an effect on photosynthetic pigment degradation, a process that in *Synechocystic* PCC6803 normally only occurs following nitrogen step-down (Richaud *et al.,* 2001). Considering that BMAA induces chlorosis in nitrogen replete cells after 24 hours, it is conceivable that a longer time frame (>12 hours) is required to observed a significant increase in PBS degradation regulatory gene expression. This however would suggest that BMAA does not have a direct effect on gene expression, as if it did the effect on gene expression levels would be more immediate. Data suggest that the effect of BMAA on PBS degradation is rather an indirect effect involving.

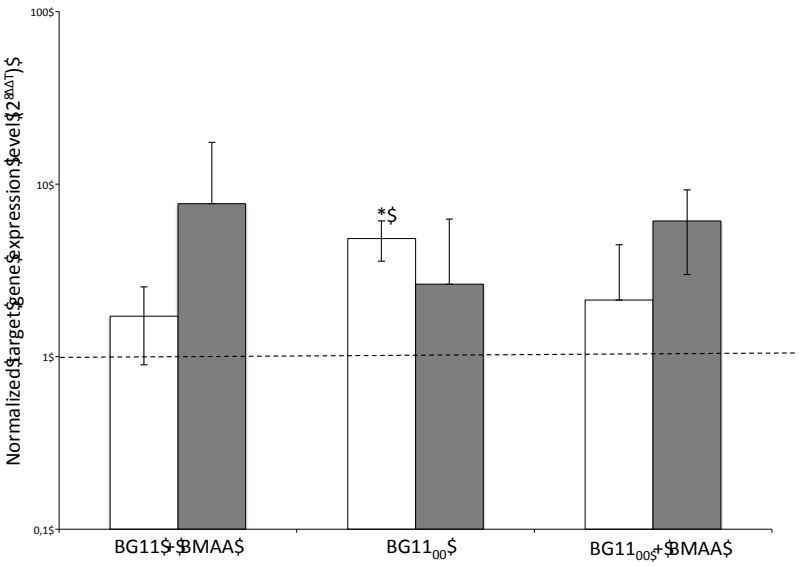

Figure 7: Gene expression levels (expressed as $\Delta\Delta C_T$) of *nbl*A1, normalized to the reference gene *16S rrna,* in *Synechocystis* PCC6803 grown in the presence and absence of BMAA (100 µM) in both N- and N+ growth medium for 6 hours (open bars) and 12 hours (grey bars). Target gene expression levels in samples were calibrated against that of cells grown in N+ (BG11) medium in the absence of BMAA (dotted line). Error bars denote standard deviations where *n*=3. Significant (p<0.05) difference in gene expression levels from control cultures is indicated by (*).

5 CONCLUSIONS

The expression of four nitrogen-controlled genes was analysed to determine whether BMAA may have a direct regulatory effect on nitrogen-controlled cellular processes at a transcriptional level. The expression of all four genes investigated was upregulated in the absence of combined nitrogen. An increase in expression, in cells grown in the absence of nitrogen, relative to control cells was only observed in two of the four genes, and only one of these observed increases was significant. This strongly suggests that the cells did not experience nitrogen starvation sufficient to induce characteristic transcriptional responses. As the transcriptional regulation of these genes is dependent on various inter-related factors such as light, redox state, reducing power and α-ketogluterate levels, it is possible that a longer time frame of starvation is required to get the cells into a state under which nitrogen-controlled regulatory processes are activated. However, whether or not the cells were adequetly nitrogen starved would only effect the accuracy of the data pertaining to the cells grown in the absence of nitrogen, and would not effect the accuracy of the data on the effect of BMAA on nitrogen-controlled gene expression in nitrogen replete cells. Based on a previous study (Downing *et al.,* 2012) that showed strong evidence that BMAA has an effect on PBS degradation even in the presence of nitrogen, a hypothesis was formulated that BMAA may have an effect of nitrogen-controlled cellular processes even in the presence of nitrogen. In the current study data showed that BMAA induced a significant increase in *ntc*A

expression in nitrogen replete cells, however, although significant this increased *ntc*A gene expression in cells grown in the presence of BMAA was only ~1.5 fold greater than that in nitrogen replete cells not exposed to BMAA and therefore not a marked increase. Although slightly increased expression of *nbl*A1 and *gln*N genes were also observed in BMAA-dosed nitrogen replete cells, these increases were also not marked nor signficant. While in some cases data suggest an effect of BMAA on gene expression, the results are not conclusive. Based on gene expression analysis of four nitrogen-controlled genes central to nitrogen regulation in *Synechocystis* PCC6803 it seems therefore that the induced effects of BMAA on nitrogen-controlled cellular responses, such as chlorosis, are not a function of direct transcriptional regulation by this amino acid. If BMAA had a direct effect of gene transcription an apparent change in the relative expression levels of selected genes involved in nitrogen-controlled cellular responses would occur more immediately and within the timeframe of this experiment and this was not observed.

The observed effects of BMAA on nitrogen-controlled cellular processes such as chlorosis, are more likely a function of BMAA disrupting the cellular carbon-nitrogen balance, which subsequently has a indirect effect of gene expression via α-ketogluterate modulation of NtcA activity. A recent study has shown that BMAA may act as a substrate for glutamate synthase (GOGAT) in the GS-GOGAT pathway (Downing, 2015), which may result in an accumulation of α-ketogluterate. A BMAA-induced shift in the carbon-nitrogen balance of the cell via non-specific enzyme binding, would induce cellular reponses characteristic to those under nitrogen starvation, and these reposnes would be induced over a longer timeframe. This hypothesis, that BMAA induces nitrogen-starvation-like responses by altering the cellular carbon-nitrogen balance is corroborated by previous studies in which BMAA induced chlorosis and quinescence was observed only after 24 hours and also by this current study in which no marked responses in nitrogen controlled gene expression was observed within a relatively shorter time frame. Further support for this hypothesis is the fact that the only significant BMAA induced increase in expresison of any of the genes investigated here was observed for NtcA. The expresison of *ntc*A is directly effected by α-ketogluterate levels and it would be expected that increased levels of α-ketogluterate would first have an effect on *ntc*A expression after which the slightly delayed effect would be observed in other NtcA regulated genes like *gln*N and *gln*B.

Investigation and analysis of the interaction of BMAA with GOGAT and the effect of BMAA of the cellular carbon-nitrogen balance and redox state and the indirect effect of BMAA on other nitrogen-controlled processes, like chlorosis, is ongoing and will increase the understanding of the physiological role of BMAA inside cyanobacteria.

6 RECOMMENDATIONS

We strongly recommend a long term BMAA monitoring project on a major water source that does experience cyanobacterial blooms so as to develop and test a simple predictive model for BMAA based on environmental combined nitrogen fluctuations and cyanobacterial biomass.

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