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Stress effects of cyanotoxin β-methylamino-L-alanine (BMAA) on cyanobacterial heterocyst formation and functionality

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Summary

Various species of cyanobacteria, diatoms and dinoflagellates are capable of synthesizing the non-proteinogenic neurotoxic amino acid β-N-methylamino-L-alanine (BMAA), which is known to be a causative agent of human neurodegeneration. Similar to most cyanotoxins, the biological and ecological functions of BMAA in cyanobacteria are unknown. In this study, we show for the first time that BMAA, in micromolar amounts, inhibits the formation of heterocysts (specialized nitrogen-fixing cells) in heterocystous, diazotrophic cyanobacteria [*Anabaena* sp. PCC 7120, *Nostoc punctiforme* PCC 73102 (ATCC 29133), *Nostoc* sp. strain 8963] under conditions of nitrogen starvation. The inhibitory effect of BMAA is abolished by the addition of glutamate. To understand the genetic reason for the observed phenomenon, we used qPCR to study the expression of key genes involved in cell differentiation and nitrogen metabolism in the model cyanobacterium *Anabaena* sp. PCC 7120. We observed that in the presence of BMAA, *Anabaena* sp. PCC 7120 does not express two essential genes associated with heterocyst differentiation, namely, *hetR* and *hepA*. We also found that addition of BMAA to cyanobacterial cultures with mature heterocysts inhibits *nifH* gene expression and nitrogenase activity.

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Introduction

Cyanobacteria are ancient, diverse microorganisms that contribute significantly to carbon and nitrogen cycles in the biosphere. It is difficult to overestimate the impact of cyanobacteria on marine primary production (Flombaum *et al.*, 2013). Cyanobacteria also synthesize a large number of secondary metabolites with different chemical structures that exhibit an exciting range of biological activities (Tan, 2007; Wase and Wright, 2008). One of these metabolites is a non-proteinogenic amino acid, β-N-methylamino-L-alanine (BMAA) that belongs to the range of cyanotoxins present in various water bodies (Metcalf *et al.*, 2008; Jonasson *et al.*, 2010). BMAA is neurotoxic to animals (including humans), causing neurodegenerative diseases such as Alzheimer's and Parkinson's diseases as well as amyotrophic lateral sclerosis (Weiss and Choi, 1988; Duncan *et al.*, 1991; Cox *et al.*, 2003; Murch *et al.*, 2004; Rao *et al.*, 2006; Xie *et al.*, 2013; Rodgers, 2014). Various species of cyanobacteria and other representatives of marine phytoplankton, including diatoms and dinoflagellates, are capable of synthesizing BMAA (Cox *et al.*, 2003; 2005; Cervantes Cianca *et al.*, 2012; Jiang *et al.*, 2014a; Lage *et al.*, 2014) at a broad range of concentrations – from 0.001 to 0.3 to approximately 1000–6000 μg g^{-1} dry weight (Cox *et al.*, 2005; Esterhuizen and Downing, 2008; Jonasson *et al.*, 2010). Different researchers independently discovered BMAA in natural waters (Al-Sammak *et al.*, 2014; Yan *et al.*, 2017) and in cyanobacteria isolated from marine and fresh waters (Metcalf *et al.*, 2008; Jonasson *et al.*, 2010; Downing *et al.*, 2011). In a marine environment such as the Baltic Sea, the BMAA levels produced by cyanobacteria were estimated to be in the range of 0.001–0.015 μg g^{-1} dry weight of cyanobacteria, and higher levels of BMAA were detected in zooplankton (0.0037–0.087 μg g^{-1} dry weight) and in different fish species (0.0019–1.29 μg g^{-1} dry weight)

(Jonasson *et al*., 2010). Thus, high amounts of BMAA may enter humans via bioaccumulation in seafood (Jiang *et al*., 2014b; Salomonsson *et al*., 2015). BMAA has diverse effects on phytoplankton, plants and animals (Popova and Koksharova, 2016). However, so far there is little information available on the biological role of BMAA in cyanobacteria.

It has been reported that BMAA might be synthesized in non-diazotrophic cyanobacterial cells in response to nitrogen starvation (Downing *et al*., 2011). Recently, the glutamine oxoglutarate aminotransferase (GOGAT) enzyme was suggested to be involved in the late stage of BMAA biosynthesis (Downing and Downing, 2016). It is possible that BMAA participates in the regulation of cyanobacterial chlorosis under conditions of nitrogen starvation, causing photoinhibition (Downing *et al*., 2015). It is known that exogenously applied BMAA significantly inhibits the growth of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 in a concentration-dependent manner (in the range of 4.2–42 μM) and causes a significant loss of photosynthetic pigments (Downing *et al*., 2012). The addition of micromolar concentrations of BMAA (20 and 50 μM) to a filamentous nitrogen-fixing cyanobacterium *Anabaena* (*Nostoc*) sp. PCC 7120 results in the inhibition of nitrogenase activity and stimulation of glycogen accumulation in vegetative cells (Berntzon *et al*., 2013). To date, the reasons behind this activity of BMAA were unknown. The goal of this study was to advance our understanding of the regulatory effect of BMAA on nitrogen fixation and heterocyst differentiation processes by using a model nitrogen-fixing cyanobacterium, *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120, hereafter referred to as *Anabaena* 7120. This cyanobacterium forms specialized cells called heterocysts that protect nitrogenase (an enzyme responsible for catalyzing nitrogen fixation) from oxygen and provide neighbouring vegetative cells with nitrogenous compounds in the form of glutamine and β-aspartyl-arginine (Flores and Herrero, 2010; Burnat *et al*., 2014; Herrero *et al*., 2016).

In this work, we asked several questions: (1) Why does BMAA inhibit nitrogen fixation in *Anabaena* 7120?; (2) Does BMAA influence heterocyst differentiation processes?; and (3) Could BMAA affect the expression of genes that control cell differentiation and nitrogen metabolism in *Anabaena* 7120? We applied fluorescence microscopy and reverse transcription polymerase chain reaction (RT-PCR) to investigate BMAA impact on these key physiological processes. In this work, we studied the effect of BMAA on the cyanobacterium *Anabaena* 7120 residing in one of two physiological states. In the first physiological state, the cyanobacterial culture contains mature heterocysts, and in the second physiological state, the culture has to form new heterocysts from vegetative cells during nitrogen deprivation.

We concluded that micromolar concentrations of exogenously added BMAA lead to drastic changes in the heterocyst differentiation process and suppress nitrogenase activity in filamentous diazotrophic cyanobacteria.

Results and discussion

Inhibition of diazotrophic cyanobacterial growth and nitrogen fixation by BMAA

First, we studied the effect of BMAA on *Anabaena* 7120 when the cyanobacterium already possessed mature heterocysts before being subjected to BMAA treatment. To examine the effect of BMAA on *Anabaena* 7120 growth we measured the optical density (OD_{750}) and chlorophyll *a* (Chl*a*) concentrations in the cyanobacterial cells, which were exposed to different concentrations of BMAA in nitrate-free liquid medium. BMAA exhibited an inhibitory, concentration-dependent effect on *Anabaena* 7120 growth over 6 days of incubation (Supporting Information Fig. S1). Addition of 50 and 100 μM BMAA led to substantial inhibition of cyanobacterial diazotrophic growth and to rapid cell lysis (Supporting Information Fig. S1). Such growth suppression could be caused by inhibition of nitrogen fixation.

To test the ability of *Anabaena* 7120 to fix atmospheric nitrogen in the presence of BMAA, the nitrogenase activity was measured by using the acetylene reduction assay (ARA) coupled with gas chromatography as described by Capone and Montoya (2001). High levels of nitrogen fixation activity were detected in the control sample (Supporting Information Fig. S2). Strong inhibition of nitrogenase activity was observed (Supporting Information Fig. S2) when *Anabaena* 7120 filaments were exposed to 20 and 50 μM BMAA. Nitrogen fixation activity decreased three-fold after incubation of cyanobacterial cells with 20 μM BMAA. The higher concentration of BMAA (50 μM) suppressed nitrogenase activity approximately 17-fold and 24-fold after 24 and 96 h of treatment respectively (Supporting Information Fig. S2). Thus, we inferred that BMAA suppressed the growth and nitrogenase activity of the cyanobacterium *Anabaena* 7120 in a culture containing mature heterocysts. This result is consistent with previously obtained results (Berntzon *et al*., 2013).

Since nitrogenase activity occurs in heterocysts, we decided to estimate the frequency of heterocyst formation in cultures treated with BMAA. Mature heterocysts appear as dark nonfluorescent cells in cyanobacterial cultures grown in $BG11_0$ medium (Supporting Information Fig. S3). These heterocysts do not exhibit autofluorescence due to reduced photosystem II (PS II)-associated antenna pigments and PS II activity

(Thomas, 1970; Donze *et al*., 1972, Kumazaki *et al*., 2013; Ferimazova *et al*., 2013; Nozue *et al*., 2016). We determined that the addition of BMAA to *Anabaena* 7120 cultures had a small effect on heterocyst frequency (Supporting Information Table S1). The heterocyst frequency was 5.1% in the control culture, whereas the heterocyst frequency reduced to 4.4% in the culture treated with 20 μM BMAA (Supporting Information Table S1). In addition, BMAA induced a slight increase in the sizes of both vegetative cells and heterocysts (Supporting Information Fig. S3B). The mean sizes of vegetative cells were 10.18 ± 1.77 μm^2 in the control samples and increased to 12.61 ± 1.84 μm^2 while under BMAA exposure ($p < 0.05$). The mean sizes of heterocysts were 12.73 ± 2.28 μm^2 and 15.31 ± 4.33 μm^2 ($p < 0.05$) in the control and BMAA-treated samples respectively. Therefore, we can conclude that the heterocyst frequency did not critically change in the presence of BMAA (Supporting Information Table S1), so the decrease in nitrogenase activity could possibly be due to nitrogenase inactivation or/and deficiency.

To identify the possible reason for decreased nitrogenase activity after BMAA treatment, we applied RT-PCR to test *nifH* gene expression in the control and BMAA-treated samples. The *nifH* gene encodes a component of nitrogenase, named dinitrogenase reductase (also called iron (Fe) protein or component II), and has traditionally been used as a marker for studying the process of nitrogen fixation (Buikema and Haselkorn, 1991; Boison *et al*., 2004; Man-Aharonovich *et al*., 2007; Vintila and El-Shehawy, 2007; Severin *et al*., 2010; Wang *et al*., 2015). Our data demonstrated that 20 μM BMAA lowered *nifH* gene expression after 48 h compared with that in the control sample and fully inhibited *nifH* gene expression after 96 h (Fig. 1), which could explain the decrease in nitrogenase activity in *Anabaena* 7120 (Supporting Information Fig. S2).

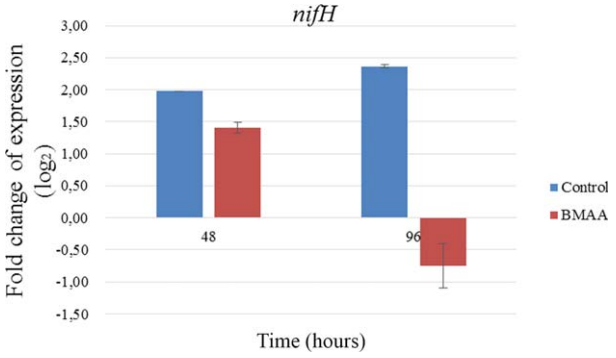

Fig. 1. Changes in the transcript levels of the *nifH* gene in mature heterocysts in *Anabaena* 7120 after the addition of 20 μM BMAA. Fold changes in the transcript levels at each timepoint compared with the transcript levels at time zero are shown as base-2 logarithm values. Error bars represent the SD of triplicate reactions.

However, what was the reason behind the *nifH* gene suppression? It is known that high concentrations of ammonium ions inhibit *nifH* gene expression (Vintila and El-Shehawy, 2007), so we hypothesized that the inhibition of *nifH* gene expression could be due to the accumulation of ammonium in heterocysts under BMAA treatment. It has been reported that ammonium ions can be accumulated in cells as a result of BMAA decomposition (Nunn, 2009; Nunn and Ponnusamy, 2009). Another possible reason for ammonium accumulation in heterocysts is the perturbance of glutamine synthetase (GS) activity caused by BMAA treatment. In diazotrophic conditions, nitrogenase converts dinitrogen to ammonium ions, which are further used by the enzyme GS to synthesize glutamine from glutamate in heterocysts. It is known that BMAA can act as analogue of glutamate (Weiss and Choi, 1988; Brownson *et al*., 2002) and can compete with glutamate for binding to glutamate receptors (Rakonczay *et al*., 1991; Rao *et al*., 2006; Cucchiaroni *et al*., 2010). Therefore, we hypothesized that BMAA can compete with glutamate to bind to GS. This misbinding of GS to BMAA (instead of glutamate) can prevent the formation of glutamine in heterocysts and can lead to excessive accumulation of ammonium ions, inhibiting *nifH* gene expression and nitrogenase activity. Further experimental studies are needed in the future to test these hypotheses.

BMAA inhibits heterocyst formation during nitrogen deprivation

As shown above, BMAA inhibits nitrogenase activity in mature heterocysts of *Anabaena* 7120, which gives rise to a new question. Could BMAA affect the formation of new heterocysts in diazotrophic filamentous cyanobacteria? To answer this question, we tested the ability of BMAA to influence the heterocyst differentiation process in *Anabaena* 7120 during nitrogen starvation. In the control culture, mature heterocysts were structurally distinguishable within 36–48 h after the start of nitrogen deprivation (Fig. 2A), and after 72 h of nitrogen starvation the heterocyst frequency was approximately 6% (Supporting Information Table S1). In the presence of 20 μM BMAA, neither mature heterocysts nor proheterocysts were seen, even after 48 and 72 h of nitrogen starvation (Fig. 2B). Evidently, BMAA suppressed heterocyst formation. Moreover, after 96 h of incubation with BMAA, many filaments were seen to be degraded, and the cyanobacterial culture was no longer viable. Apparently, a starving cyanobacterial culture that is devoid of heterocysts dies. However, the addition of glutamate at different concentrations (0.05–3 mM) simultaneously with 20 μM BMAA abolished the inhibitory effect of BMAA on cell differentiation: mature heterocysts were

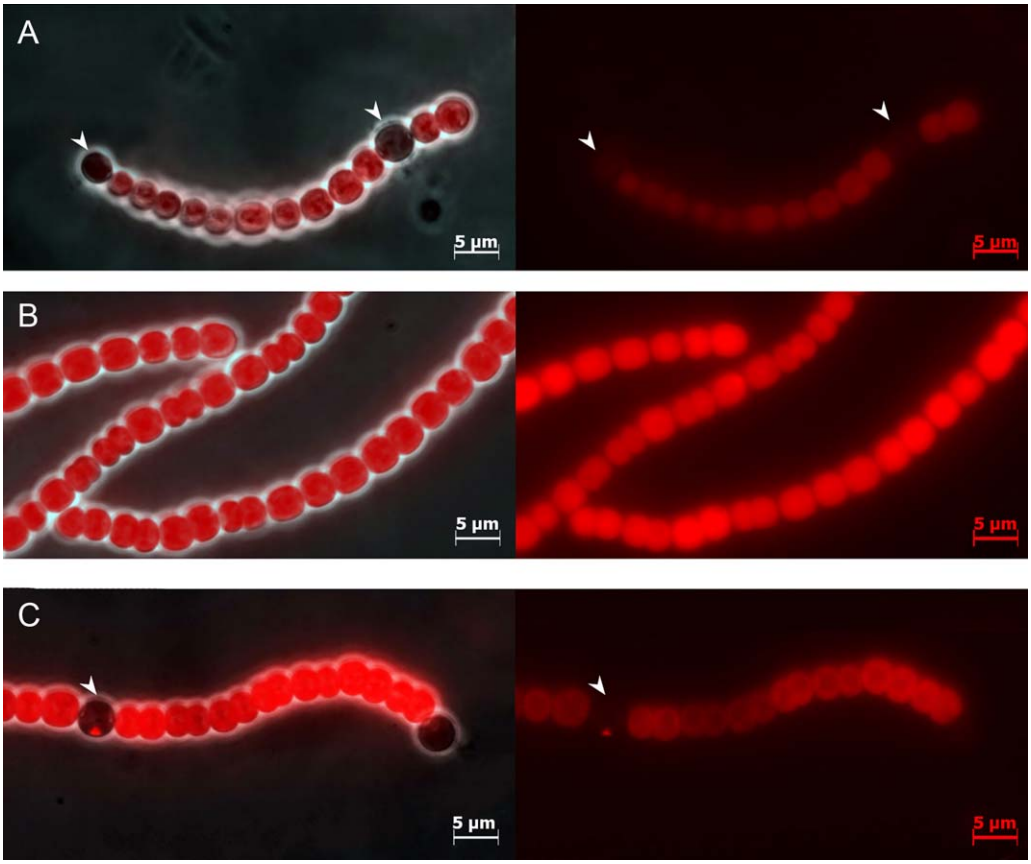

Fig. 2. Microphotographs of *Anabaena* 7120 filaments after 72 h of nitrogen deprivation. The panels on the left show the filaments in the light-field combined with fluorescent images. The panels on the right show chlorophyll autofluorescence.
A. *Anabaena* 7120 in the $BG11_0$ medium without BMAA addition.
B. *Anabaena* 7120 after 72 h of growth in the presence of 20 μM BMAA.
C. *Anabaena* 7120 after 72 h of growth with 20 μM BMAA and 250 μM glutamate. Heterocysts are marked with arrows.

observed in *Anabaena* 7120 (Fig. 2C), and heterocyst frequencies in the presence of glutamate were similar to the heterocyst frequency in the control sample (Supporting Information Table S1). This observation may largely be a result of the inhibition by glutamate of BMAA uptake, which may occur via the known glutamate transporters of *Anabaena* 7120 (Pernil *et al*., 2008).

A similar biological effect of BMAA on heterocyst formation has been detected in two other diazotrophic filamentous cyanobacteria: *Nostoc punctiforme* PCC 73102 (ATCC 29133) (Supporting Information Table S2) and *Nostoc* sp. strain 8963 (Supporting Information Fig. S4). Both strains form symbiotic relationships with plants. *Nostoc punctiforme* PCC 73102 forms a symbiotic association with the cycad *Macrozamia* sp. (Rippka *et al*., 1979), and *Nostoc* sp. strain 8963 forms a symbiotic association with the flowering plant *Gunnera prorepens* (Cox *et al*., 2005). Thus, we found that BMAA had an inhibitory effect on heterocyst formation in different filamentous nitrogen-fixing cyanobacteria.

To investigate the genetic reason for the observed effect of BMAA, we then analyzed the expression of several key genes involved in heterocyst development and function (*hetR*, *hepA*, *nifH*, *ntcA*) and in nitrogen assimilation (*glnA*, *gltS* and *nirA)* in the model cyanobacterium *Anabaena* 7120.

BMAA suppresses genes involved in heterocyst formation and functionality and does not alter the expression of several genes involved in nitrogen assimilation

The expression of genes enabling differentiation of vegetative cells into heterocysts starts during nitrogen step-down (Buikema and Haselkorn, 1991; Golden *et al*., 1991; Black *et al*., 1993; Herrero *et al*., 2004; Thiel, 2005; Sarma, 2012). We used quantitative RT-PCR (Table 1; Supporting Information Table S3) to evaluate the transcription levels of genes (*hetR*, *hepA*, *nifH*, *ntcA*) responsible for heterocyst formation or functionality in *Anabaena* 7120 under conditions of nitrogen starvation.

Table 1. qRT-PCR analysis of the expression of nitrogen-regulated genes in the absence (control) or presence of 20 μM BMAA after 4, 8, 24 and 48 h of nitrogen starvation in *Anabaena* 7120.

Gene	Product	Culture condition	Fold change in expression (log_2)*			
			4 h	8 h	24 h	48 h
hetR	Heterocyst differentiation protein HetR	Control	**1.19 ± 0.07**	**3.03 ± 0.13**	**1.33 ± 0.08**	**2.11 ± 1.10**
		BMAA	**−0.06 ± 0.03**	**−0.16 ± 0.04**	**0.07 ± 0.14**	**−0.13 ± 0.12**
hepA	Heterocyst differentiation protein HepA	Control	−1.42 ± 0.10	**3.56 ± 0.39**	**2.72 ± 0.07**	**4.99 ± 0.06**
		BMAA	−0.75 ± 0.08	**0.70 ± 0.39**	**1.46 ± 0.52**	**0.44 ± 0.01**
ntcA	Nitrogen-responsive regulatory protein NtcA	Control	1.27 ±0.05	**2.77 ±0.65**	1.47 ±0.08	**3.11 ±0.18**
		BMAA	0.77 ±0.24	1.21 ±0.18	2.12 ±0.08	**0.71 ±0.24**
nifH	Nitrogenase subunit	Control	−0.75 ± 0.12	1.25 ± 0.11	3.21 ± 0.38	**8.13 ± 0.30**
		BMAA	0.91 ± 0.13	−0.19 ± 0.51	2.09 ± 0.65	**3.25 ± 0.11**
glnA	Glutamine synthetase	Control	−0.72 ± 0.81	0.56 ± 0.17	**1.79 ± 0.22**	−1.03 ± 0.15
		BMAA	−1.32 ± 0.09	1.44 ± 0.20	**−1.53 ± 0.55**	0.71 ± 0.01
gltS	Glutamine-oxoglutarate-aminotransferase	Control	−0.58 ± 0.35	0.51 ± 0.31	−1.51 ± 0.12	−0.68 ± 0.61
		BMAA	−1.49 ± 0.33	−0.88 ± 0.02	−0.75 ± 0.03	0.10 ± 0.30
nirA	Nitrite reductase	Control	0.15 ± 0.07	−0.26 ± 0.83	1.37 ± 0.84	1.13 ± 0.09
		BMAA	−0.23 ± 0.03	0.76 ± 0.11	1.07 ± 0.44	2.08 ± 1.16

*Fold changes in expression are reported as log_2 values. Each sample was measured in triplicate, and the standard deviation is indicated by error bars. Values were normalized to the *rnpB* transcript level. Transcript levels between the control and treated samples were tested by Student's *t*-test ($p < 0.05$), and significant differences are shown in bold. Gene-specific primers are listed in Supporting Information Table S3.

It was found that addition of 20 μM BMAA strongly inhibited the expression of two key genes, *hetR* and *hepA*, whose protein products are essential for heterocyst formation (Table 1).

It is known that in cyanobacteria, HetR (a master control protein specific to heterocyst differentiation) interacts with the promoter of the *hepA* gene and regulates the expression of this gene. The *hepA* gene encodes an ABC-type transporter that is essential for the synthesis of the polysaccharide capsule of heterocysts (Black *et al*., 1993; Leganés, 1994; Du *et al*., 2012; Videau *et al*., 2014). In the control culture of *Anabaena* 7120, *hetR* gene expression was upregulated within 4 h after the start of nitrogen deprivation and peaked at 8 h. However, in the presence of BMAA, *hetR* gene expression was repressed (Table 1). Normally *hetR* expression is followed by expression of the *hepA* gene in proheterocysts (Black *et al*., 1993; Muro-Pastor and Hess, 2012). In the control sample, *hepA* expression increased in response to *hetR* upregulation within 8 h of nitrogen step-down (Table 1). This result was consistent with the data described in Black *et al*. (1993), Huang *et al*. (2004) and Sarma (2012). In contrast, we found that in the presence of BMAA, *hepA* gene expression was also repressed (Table 1). This result is consistent with the repression of *hetR* gene expression. The products of the *hetR* and *hepA* genes are essential for heterocyst differentiation; thus, inhibition of the transcription of these genes could explain why *Anabaena* 7120 did not form heterocysts in the presence of BMAA (Fig. 2B).

In the final stages of heterocyst formation, subunits of nitrogenase are synthesized, which requires transcription and DNA rearrangement of the *nifHDK* operon (Golden *et al*., 1991). In our experiments, *nifH* gene expression under BMAA treatment was not upregulated compared with that in the control sample (Table 1). The lack of *nifH* gene expression can be explained by the disruption of later stages of functional heterocyst differentiation, since BMAA inhibited *hetR* and *hepA* gene expression earlier.

The *ntcA* gene encodes NtcA, which is a global control protein for nitrogen metabolism and a transcription factor that belongs to the cAMP receptor protein family (Herrero *et al*., 2004). In the control sample, the level of *ntcA* transcription increased at 8 and 48 h after the start of nitrogen deprivation, whereas BMAA retarded the upregulation of *ntcA* and did not significantly affect the expression of this gene during the first 24 h in cyanobacterial cells (Table 1).

We also examined the effect of BMAA influence on the expression of the *glnA*, *gltS* and *nirA* genes, which encode three additional nitrogen-metabolism-associated enzymes (Table 1). Nitrogen assimilation in vegetative cells requires the activity of GS and GOGAT, which are encoded by the *glnA* and *gltS* genes respectively. The gene *glnA* is expressed in both vegetative cells and heterocysts (Tumer *et al*., 1983; Frías *et al*., 1994; Thiel, 2005). In our experiment, during the first 8 h after the onset of nitrogen step-down, when the process of heterocyst differentiation is initiated, the expression of the *glnA* gene was not significantly different between the control sample and the BMAA-treated sample. In addition, BMAA did not noticeably change the transcript levels of the *gltS* and *nirA* genes (Table 1). The *nirA* gene encodes nitrite reductase, one of the main enzymes involved in nitrate assimilation (Luque *et al*., 1993;

Cai and Wolk, 1997). GOGAT and nitrite reductase activities are usually almost absent in heterocysts (Thomas *et al*., 1977; Martin-Figueroa *et al*., 2000; Frías and Flores, 2015), which is consistent with the low transcript levels of the *gltS* and *nirA* genes observed in the control culture during nitrogen deprivation (Table 1). The absence of a noticeable effect of BMAA on the expression of these genes (Table 1) likely indirectly reflects the fact that the main targets of BMAA activity are the processes specifically associated with heterocyst differentiation. The expression of the 'housekeeping' gene *rnpB* was not affected by the action of BMAA (data not shown).

One of the most interesting questions is the following: how does BMAA affect *hetR* gene expression?

We hypothesize that there could be several reasons for BMAA-induced *hetR* gene repression. One of these reasons is that BMAA can disrupt the proper binding of 2-oxoglutarate (2-OG) to NtcA. Previously, it has been experimentally shown that the signaling molecule 2-OG is involved in the signalling response to nitrogen stress (Li *et al*., 2003; Zhang *et al*., 2006; Muro-Pastor and Hess, 2012). Thus, heterocyst frequency rises with increasing 2-OG concentrations in the growth medium (Li *et al*., 2003). 2-OG increases binding affinity of the protein NtcA for some of the target promoters of NtcA (Vázquez-Bermúdez *et al*., 2002; Zhao *et al*., 2010). It is also known that the NtcA and HetR proteins positively regulate their own expression as well as each other's expression over the course of the heterocyst differentiation (Herrero *et al*., 2004; Muro-Pastor and Hess, 2012; Herrero *et al*., 2016). It is known that 2-OG can be metabolized via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway (Muro-Pastor *et al*., 2005; Luque and Forchhammer, 2008; Domínguez-Martín *et al*., 2014). However, BMAA perturbs the metabolism of amino acids in eukaryotic cells, including the metabolic pathways of alanine, aspartate and glutamate (Nunn and Ponnusamy, 2009; Engskog *et al*., 2017). Therefore, if BMAA, acting as an analogue of glutamate, disrupts the functioning of these pathways in cyanobacteria, BMAA could impair nitrogen control by competing with natural signals such 2-OG and could cause incorrect regulation of *hetR* gene expression. Verification of this hypothesis requires further study.

Based on our data, the results obtained by Downing *et al*. (2011) could be interpreted from a new perspective. Hypothetically, the enhancement of BMAA biosynthesis by non-nitrogen-fixing cyanobacteria during nitrogen starvation (Downing *et al*., 2011) is likely an antagonistic tool against nitrogen-fixing cyanobacteria competing for supplies under conditions of nitrogen starvation. Since synthesis of BMAA depends on environmental conditions, the specific ecological roles of BMAA can be associated with the regulation of nitrogen metabolism in cyanobacteria and with the operation of the adaptive cell response to the N/C balance in the medium.

Notably, other representatives of marine phytoplankton, such as diatoms and dinoflagellates, which are able to synthesize BMAA (Cox *et al*., 2005; Jiang *et al*., 2014a; Lage *et al*., 2014), can also use this non-proteinogenic amino acid to acquire competitive advantages.

In conclusion, in this study, we report the discovery that the cyanotoxin β-N-methylamino-L-alanine (BMAA) has important biological effects on the cyanobacterial development process and nitrogen fixation by influencing the expression of specific genes. This finding provides fresh perspective for elucidation of the possible ecological role of this neurotoxic non-proteinogenic amino acid in microbial communities and offers opportunities for new studies of microbial interactions in ecosystems. The data obtained in this work are of great importance for further fundamental studies of the molecular mechanisms underlying the regulation of nitrogen metabolism and cell differentiation in nitrogen-fixing cyanobacteria.

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Author contributions

A.A.P. contributed to data collection, data analysis and data interpretation and to writing the manuscript. U.R. contributed to data collection and to writing the manuscript. T.A.S. contributed to the design of the RT-PCR experiments and to data analysis. V.M.G. contributed to data analysis and to writing the manuscript. O.A.K. contributed to the design of the research; to data collection, data analysis and data interpretation; and to writing the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. *Anabaena* 7120 heterocyst frequency after 72 h of growth without and with BMAA addition (mean ± SD).

Table S2. *Nostoc punctiforme* PCC 73102 (ATCC 29133) heterocyst frequency after 72 h of growth with different concentrations of BMAA (mean ± SD).

Table S3. Primers used for qRT-PCR.

Fig. S1. Effect of externally added BMAA on *Anabaena* 7120 growth in diazotrophic conditions (mean ± SD). Cells were incubated with 20, 50 and 100 μM BMAA.

A. Growth was characterized as the optical density at 750 nm and (B) Chl*a* concentration (μg ml^{-1}).

Fig. S2. Effect of BMAA on the nitrogen fixation rate of *Anabaena* 7120 (mean ± SD). Cells were grown in BG11$_0$ in the presence of 0, 20 and 50 μM BMAA. The nitrogen fixation rate was measured after 48 and 96 h of BMAA treatment.

Fig. S3. Microphotographs of *Anabaena* 7120 filaments after 72 h of growth. The panels on the left show the filaments in lightfield combined with fluorescent images. The panels on the right show the chlorophyll autofluorescence. Heterocysts do not show fluorescence.

A. Control sample of *Anabaena* 7120.B. *Anabaena* 7120 culture after 72 h of exposure to 20 μM BMAA. Heterocysts are marked with arrows.

Fig. S4. Microphotographs of *Nostoc* sp. strain 8963 filaments after 48 h of nitrogen deprivation. The panels on the left show the filaments in the lightfield combined with fluorescent images. The panels on the right show the chlorophyll autofluorescence.

A. *Nostoc* sp. strain 8963 without BMAA treatment.

B. *Nostoc* sp. strain 8963 after 48 h of growth with 50 μM BMAA. Heterocysts are marked with arrows.