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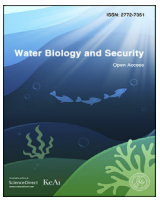

Research Article

Investigation on cyanobacterial production of the proposed neurotoxin β-N-methylamino-L-alanine (BMAA)

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ABSTRACT

β-N-methylamino-L-alanine (BMAA) is an environmental neurotoxin thought to be produced by cyanobacteria. However, the cyanobacterial origin of BMAA remains controversial. The detection method and culture conditions of cyanobacteria are often cited as factors behind the discrepancy of published results. We showed previously that BMAA was highly toxic to the cyanobacterium *Nostoc* PCC 7120, and it is taken up via an amino acid transport system. Using a mutant Δ*natA*Δ*bgtA* deficient in amino acid transport as a genetic control, we show here that BMAA taken up from the medium can be detected quantitatively at a threshold similar to, or below those reported, but was undetectable in the mutant. The BMAA isomer, 2,4-diaminobutanoic acids (DAB), but not BMAA, could be detected in cell free extracts of *Nostoc* PCC 7120. Long-term (20 days) diazotrophic growth or non-limiting supply of phosphate, conditions reported to enhance BMAA synthesis, did not lead to the detection of BMAA. An UPLC-MS/MS signal with a similar retention time to BMAA was found after prolonged diazotrophic incubation, but did not have fragment ions of BMAA after further analysis. When extended to 29 different cyanobacterial strains and 6 natural cyanobacterial bloom samples, none of them was found to produce BMAA. The cytotoxicity of BMAA to cyanobacteria, and the lack of a cellular protective mechanism against such toxicity, contradict the presence of a BMAA synthesis pathway in these organisms. More specific methods for BMAA detection in vivo need to be developed to clarify the cyanobacterial origin of BMAA.

1. Introduction

β-N-methylamino-L-alanine (BMAA) is a non-protein amino acid, first identified from Cycas in 1967 (Vega and Bell, 1967). The studies on BMAA gained considerable interests since many reports pointed to a correlation between BMAA and neurodegenerative diseases such as amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease (Nunn et al., 1987; Delcourt et al., 2017). Furthermore, a number of studies indicated that BMAA was produced by cyanobacteria (Nunn, 2017). One starting hypothesis suggests that BMAA produced from cyanobacteria in symbiosis with cycad accumulates through the food chain in brain tissue of flying foxes and humans, and constitutes a causal factor for the high incidence of neurodegenerative diseases among the indigenous population in Guam (Cox et al., 2003). Since cyanobacteria are widespread in the environment, BMAA is thought to be an environmental neurotoxin. However, both the production of BMAA, and BMAA as a cause of neurodegenerative diseases remain controversial issues since the data is often contradictory, and even the detection of BMAA itself in biological samples including cyanobacteria is often contested (Chernoff et al., 2017; Rauk, 2018; Zguna et al., 2019; Dunlop et al., 2021). Indeed, there is no standard detection technique, and even the same detection method can yield different results from different laboratories (Faassen et al., 2012; Faassen, 2014; Lage et al., 2016; Baker et al., 2018; Banack and Murch, 2018; Manolidi et al., 2018; Bishop and Murch, 2019; Banack, 2021; Tymm et al., 2021). BMAA has two described forms: free BMAA and protein-associated BMAA, and the

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nature of the latter remains unclear (Rosen et al., 2016; Beach et al., 2018). When detection is positive, the amount of BMAA reported from cyanobacteria varies considerably, ranging from 0.05 to about 6500 μg/g DW (dry weight) for free BMAA, and 0.02 to 5500 μg/g DW for protein-associated BMAA (Manolidi et al., 2018). Examples of negative results were also reported with no BMAA detected from cyanobacterial samples (Kubo et al., 2008; Rosen and Hellenas, 2008; Moura et al., 2009; Kruger et al., 2010; Li et al., 2012; Réveillon et al., 2014; Fan et al., 2015). As an amino acid analog, it has no chromophore or fluorophore for analytical detection. Therefore, a derivatization step is often necessary to enable detection. The most frequently used derivatization reagent was 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Manolidi et al., 2018; Bishop and Murch, 2019). High or ultra-high performance liquid chromatography with tandem mass spectrometry detection (UPLC-MS/MS), combined with derivatization using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), is the most frequently used technique for BMAA detection (Manolidi et al., 2018; Bishop and Murch, 2019). This method relies on the retention time and characteristic fragment ions for the identification of BMAA, and is considered as one of the most reliable techniques to detect BMAA in biological samples (Spacil et al., 2010; Faassen, 2014; Lage et al., 2016; Lance et al., 2018). In addition, a few studies used propyl chloroformate or dansyl chloride as derivatization reagent (Esterhuizen and Downing, 2008; Salomonsson et al., 2013; Downing et al., 2014; Lage et al., 2016), and some studies reported the detection of BMAA without derivatization (Faassen et al., 2012; Foss et al., 2018). However, no matter what method is used for testing, some structural isomers co-occur with BMAA in cyanobacteria, which increases the difficulty of BMAA identification. The most common isomer of BMAA is 2, 4-diaminobutyric acid (DAB), which has an aminomethyl group, instead of methylamino group, linked to the β-carbon atom of L-alanine (Fig. 1) (Spacil et al., 2010). Under our assay conditions using UPLC-MS/MS, the limit of detection (LOD) of BMAA standard was 0.017 pmol per injection (or 0.5 μg/L), and the limit of quantification (LOQ) was 0.042 pmol per injection (or 1.25 μg/L) (Wang et al., 2020). The LOD determined in our experiments is amongst the lowest compared to other reported results which range from 0.016 pmol to 65 μmol per injection, except one laboratory which reported an even lower LOD at 0.01 pg (8.5×10^{-5} pmol) per injection (Baker et al., 2018; Manolidi et al., 2018).

The biochemical basis for BMAA production remains unknown (Li et al., 2023). It was reported that some cyanobacterial species and strains, namely *Synechococcus* sp. TAU-MAC 0499, *Synechocystis* PCC 6803 and *Nostoc* PCC 7120, were sensitive to exogenously added BMAA in the culture medium (Downing et al., 2012; Berntzon et al., 2013; Vergou et al., 2020). Our previous studies further indicated that BMAA could enter cyanobacterial cells via amino acid uptake systems (Wang et al., 2020). *Nostoc* PCC 7120 is a filamentous cyanobacterium that can use nitrate, ammonium and atmospheric N_2 fixed in heterocysts as nitrogen sources. Upon deprivation of fixed forms of nitrogen, heterocysts will be differentiated for nitrogen fixation (Zhang et al., 2006). Irrespective of the growth mode, *Nostoc* PCC 7120 can take up various amino acids through four ABC (ATP binding cassette)-type amino acid transporters: N–I, N-II, Bgt, and N-III. N-II and Bgt systems share a common component, the BgtA protein (Picossi et al., 2005; Pernil et al., 2008, 2015). Among these four systems, N–I, N-II and Bgt together are responsible for more than 98% of the amino acid transport activity, while the N-III plays a minor role in amino acid uptake, since its inactivation affected only weakly the uptake rate of various amino acids tested in *Nostoc* PCC 7120 (Pernil et al., 2008, 2015). We created a double mutant, Δ*natA*Δ*bgtA*, which abolished the three major amino acid transport systems in *Nostoc* PCC 7120, N–I, N-II and Bgt (Wang et al., 2020). While the wild type could uptake BMAA and was unable to grow in the presence of 25 μM of BMAA, the mutant lost the ability to uptake BMAA and gained resistance to BMAA at a concentration as high as 100 μM.

Initially, as a research project, we sought to determine the biosynthetic pathway of BMAA production in cyanobacteria through genetic approaches using model cyanobacteria, or through biochemical screening by relying on strains that were able to produce BMAA. Despite the sensitivity of the method we used (Wang et al., 2020), we were unable to detect BMAA from cyanobacteria, so we sought to validate the detection method we used. In this study, by using UPLC-MS/MS coupled with AQC derivatization, and the amino acid transport mutant Δ*natA*Δ*bgtA* as a genetic control (Wang et al., 2020), we detected no BMAA, but did detect the BMAA isomer DAB, from *Nostoc* PCC 7120 samples. Similar results were also found with other strains and bloom samples. Based on our results, we propose that the production of BMAA in cyanobacteria needs further studies.

2. Materials and methods

2.1. Cyanobacteria strains and growth conditions

Unless otherwise specified, all the strains used in this study were grown in the mineral medium BG11 as described previously (Zhang, 1993). Briefly, cyanobacterial strains were cultured in BG11 medium with constant illumination of 30 μmol $m^{-2}{\cdot}s^{-1}$. The cultures in flasks were shaken at 180 rpm. To investigate the effects of prolonged nitrogen limitation and phosphate oversupply on the biosynthesis of BMAA, *Nostoc* PCC 7120 was cultured in $BG11_0$ (same as BG11 but free of combined nitrogen) and 10P (BG11 with the concentration of K_2HPO_4 increased by 10-fold from 0.175 mM in standard BG11 medium to 1.75 mM) for 20 days. One hundred millilitres of cell cultures were collected through filtration for BMAA detection. To study whether nitrogen limitation with shorter incubation time can induce the production of BMAA in *Nostoc* PCC 7120, cells were first cultured to logarithmic phase in BG11, and 200 mL cell cultures were collected by filtration (sample time $t = 0$). Then cells were transferred to nitrate-free $BG11_0$ media, and 200 mL samples from the cultures were collected after 12 h and 24 h, respectively. For growth curve measurements, *Nostoc* PCC 7120 was cultivated in liquid medium (BG11, $BG11_0$ or 10P with two replicates) under artificial illumination (constant light, 30 μmol $m^{-2}{\cdot}s^{-1}$) with shaking at 180 rpm. The cell concentration was determined at OD_{750}.

2.2. Sample preparation for BMAA detection

At late exponential phase, 200 mL cultures (nearly 30 mg DW cells) of *Nostoc* PCC 7120 cells were harvested through filtration and used to detect BMAA. Fifty-three to 1100 mg DW cells of other cyanobacteria

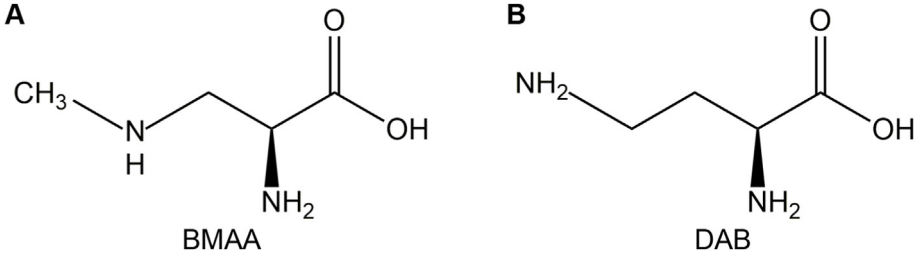

Fig. 1. Chemical structures of β-N-methylamino-L-alanine (BMAA) (**A**) and 2, 4-diaminobutyric acid (DAB) (**B**).

cultures, or from field collected algal blooms were tested for detection of BMAA. The field samples were collected by HydroBios net from Taihu Lake (31°24′36″ N, 120°11′24″ E) and Dianchi Lake. (24°41′58.00″N,102°39′53.00″E) in China. After freeze-drying, the field samples were stored at −20 °C. The details are presented in Table 1 and Table 2. Sample preparation was performed as previously described (Wang et al., 2020). Briefly, the harvested cells and ground cycad seed (collected from Wuhan, China) were boiled in 500 μL double distilled water for 20 min, and lysed using a FastPrep-24TM5G (MP Biomedicals, LLC, Santa Ana California, USA) crusher at 6 m/s for 2 min. After centrifugation (13,800 $g \times 30$ min), the supernatant was transferred to new tubes. Then 6M HCl was added into the supernatant to a final concentration of 20 mM and centrifuged again (13,800 $g \times 15$ min). The precipitate was discarded and the supernatant was stored at −80 °C before further processing.

Table 1
Detail information of 29 cyanobacteria samples used for BMAA detection.

No.	Code in FACHB	Species	Cells (for BMAA extraction (g, DW))	BMAA (ug/g DW)
1	FACHB-973	*Nostoc* sp.	0.484	n.d.
2	FACHB-1042	*Nostoc* sp.	0.066	n.d.
3	FACHB-1043	*Nostoc* sp.	0.213	n.d.
4	FACHB-1053	*Nostoc* sp.	1.010	n.d.
5	FACHB-1135	*Nostoc* sp.	0.418	n.d.
6	FACHB-1145	*Nostoc* sp.	0.461	n.d.
7	FACHB-1154	*Nostoc* sp.	0.067	n.d.
8	FACHB-1155	*Nostoc* sp.	1.180	n.d.
9	FACHB-1159	*Nostoc* sp.	0.147	n.d.
10	FACHB-1162	*Nostoc* sp.	0.202	n.d.
11	FACHB-1149	*Nostoc* sp.	1.000	n.d.
12	FACHB-259	*Nodularia harveyana*	0.461	n.d.
13	FACHB-1490	*Nodularia harveyana*	0.865	n.d.
14	FACHB-625	*Scytonema* sp.	0.069	n.d.
15	FACHB-626	*Scytonema* sp.	0.063	n.d.
16	FACHB-628	*Scytonema* sp.	0.097	n.d.
17	FACHB-633	*Scytonema* sp.	0.045	n.d.
18	FACHB-887	*Scytonema javanicum*	0.260	n.d.
19	FACHB-161	*Phormidium ambiguum*	1.100	n.d.
20	FACHB-239	*Phoridium foveolarum*	1.078	n.d.
21	FACHB-723	*Phormidium mucicola*	0.084	n.d.
22	FACHB-886	*Phormidium tenue*	0.126	n.d.
23	FACHB-1050	*Phormidium tenue*	0.290	n.d.
24	FACHB-1099	*Phormidium* sp.	0.800	n.d.
25	FACHB-1129	*Phormidium* sp.	0.060	n.d.
26	FACHB-1136	*Phormidium* sp.	0.465	n.d.
27	FACHB-1137	*Phormidium* sp.	0.068	n.d.
28	FACHB-1388	*Anabaena* sp.	0.059	n.d.
29	PCC7806	*Microcystis aeruginosa*	0.056	n.d.

n.d. denotes concentration was below the limit of detection(<LOD).

Table 2
Detail information of 6 algal bloom samples used for BMAA detection.

Sample name	Dominant species	Collection location and mm/yyyy	Cells (for BMAA extraction (g, DW))	BMAA (ug/g DW)
Algal bloom sample 1	*Microcystis*	Taihu, July 2017	0.055	n.d.
Algal bloom sample 2	*Microcystis*	Taihu, 06/2016	0.314	n.d.
Algal bloom sample 3	*Microcystis*	Dianchi, 05/2016	0.204	n.d.
Algal bloom sample 4	*Microcystis*	Dianchi, 06/2015	0.080	n.d.
Algal bloom sample 5	*Aphanizomenon*	Dianchi, 03/2015	0.053	n.d.
Algal bloom sample 6	*Aphanizomenon*	Dianchi, 02/2015	0.185	n.d.

n.d. concentration was below the limit of detection(<LOD).

2.3. BMAA and DAB detection

The most frequently used technique, High or Ultra-High Performance Liquid Chromatography with tandem mass spectrometry detection (UPLC-MS/MS), combined with derivatization using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) was used in this study for BMAA and DAB detection. All the samples were derivitized using AccQ-Tag Ultra Derivatization Kit (Waters Corporation, Milford, Massachusetts, U.S.A) following the manufacturer's instructions. UPLC-MS/MS was carried out by ACQUITY UPLC H-class-Xevo TQ MS system, equipped with an ACQUITY UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm) (Waters Corporation). Solvent A (0.1% formic acid in water) and Solvent B (0.1% formic acid in methyl alcohol) were used as a gradient elution buffer with flow velocity 0.15 mL/min. The gradient elution conditions were: 0.0 min = 80% A; 4.0 min = 85% A; 4.1 min = 80% A; 5.8 min = 80% A. The Column temperature was 40 °C. The mass spectrometer conditions were as follows: electrospray mode: ESI+; Capillary (kV): 3.0; desolvation temperature: 350 °C; nitrogen gas flow desolvation (L/h): 650; argon collision gas flow (mL/min): 0.12; source temperature: 150 °C. Ion transitions were monitored by MRM (multiple reaction monitoring). 459.10 > 119.10, 459.10 > 171.10, 459.10 > 258.10 and 459.10 > 289.10 for BMAA. 459.10 > 119.00, 459.10 > 171.00, 459.10 > 145.00 and 459.10 > 315.00 for DAB. The details for sample derivatization and the determination of the limit of detection (LOD) and limit of quantification (LOQ) were described by Wang et al. (2020).

2.4. Stability of BMAA in Nostoc PCC 7120

To prepare the samples for detecting BMAA stability in cells, *Nostoc* PCC 7120 were incubated with 50 μM BMAA (purchased from Wuhan Chuanliu Biotechnology Co., Ltd.) for 20 min, and then washed three times with BMAA-free medium by filtration and resuspension. Twenty millilitres of cell cultures were harvested by filtration respectively after 0, 10, 30 and 60 min. Sample preparation, derivatization, and detection were the same as described above for the analysis of BMAA.

2.5. Statistical analysis

The growth curves were determined with two parallel cultures and presented as mean values. To show the stability of BMAA in *Nostoc* PCC 7120, data were collected from 3 biological replicates and presented as mean ± S.D. (standard deviation).

3. Results

3.1. Detection of DAB and BMAA in Nostoc PCC 7120

Previously, we applied our sensitive detection method (UPLC-MS/MS coupled with AQC derivatization) for identification of BMAA from cells of *Nostoc* PCC 7120 (Wang et al., 2020). While BMAA taken up from the growth medium was detected, no BMAA was found in *Nostoc* PCC 7120 extract from cells (Wang et al., 2020). To further validate our detection method, BMAA and DAB standards were identified successfully (Fig. 2A), and BMAA was found from extracts of cycad seeds, with a BMAA concentration of 5.5 μg/g DW (Fig. 2B). Despite the success of the control experiments, no BMAA signal was identified from cells cultured in the presence of nitrate as a combined nitrogen source (time 0, Fig. 2C).

A number of structural isomers of BMAA have been reported, including 2,4-diaminobutyric acid (DAB), 2,3-diaminobutanoic acid, 3,4-diaminobutanoic acid, N-(2-aminoethyl)glycine (AEG), β-amino-N-methylalanine (BAMA), 3-amino-2-(aminomethyl)propanoic acid and 2,3-diamino-2-methylpropanoic acid (Jiang et al., 2012; Manolidi et al., 2018). UPLC-MS/MS coupled with AQC derivatization can distinguish BMAA from DAB, AEG and BAMA (Jiang et al., 2012). Under our assay conditions, we also succeeded in distinguishing the most similar isomer DAB from BMAA using the same technique (Fig. 2A). Furthermore, when BMAA and DAB, alone or in combination, were added to cell free extracts of *Nostoc* PCC 7120, these two compounds were separated with different retention times (Fig. 2D).

3.2. DAB accumulation under nitrogen limitation

It was reported that nitrogen starvation could enhance the production of BMAA in cyanobacteria (Downing et al., 2011; Yan et al., 2020). We thus tested whether BMAA could be detected from cell free extracts of *Nostoc* PCC 7120 cultured under conditions of nitrogen limitation. For that purpose, *Nostoc* PCC 7120 was first cultured in BG11 containing nitrate as combined nitrogen (time 0), and then transferred to nitrate-free $BG11_0$ for 12 h and 24 h. As shown in Fig. 2C, no signal corresponding to BMAA was found in the UPLC-MS/MS chromatograms (Fig. 2C). However, 2, 4-DAB (an isomer of BMAA) was detected from the cell free extract of *Nostoc* PCC 7120, and its relative amount increased by 3-fold after combined-nitrogen starvation. Thus, 2,4-DAB, but not BMAA, was detected in *Nostoc* PCC 7120 and its production increased in response to combined-nitrogen starvation.

To further validate the identification of BMAA and DAB in cell free extracts of *Nostoc* PCC 7120, single ion chromatograms of both compounds were analyzed (Fig. 3). For BMAA and DAB standards, 4 fragment ions used for characterization of BMAA, and 2 for DAB, are all present (Fig. 3A). For cell free extracts prepared from N-limited *Nostoc* PCC 7120 cultures after 0 h (Figs. 3B) and 24 h (Fig. 3C), the single ion chromatograms were identical to DAB but none corresponded to BMAA. These analyses further confirmed the presence of DAB, but the absence of BMAA in the *Nostoc* PCC 7120 cultures.

3.3. Effect of replete phosphate and prolonged culturing on BMAA production

It has been reported that increasing supply of phosphate in the culture medium, or prolonged incubation under diazotrophic growth could stimulate BMAA synthesis in some cyanobacteria (Yan et al., 2020). We increased phosphate concentration by 10-fold, from 0.175 mM in standard BG11 medium to 1.75 mM, and cultured *Nostoc* PCC 7120 in $BG11_0$ medium for 20 days. Compared to the sample cultured in standard BG11 medium, the supply of a high level of phosphate in the culture medium did not significantly affect the growth of *Nostoc* PCC 7120, only delayed the entry into stationary phase, similarly to the cultures grown in $BG11_0$ (Fig. 4A).

No BMAA was identified from the prolonged culture in BG11 or in high phosphate. A peak corresponding to DAB was found from the samples prepared from cells with prolonged incubation in $BG11_0$ and high phosphate. Interestingly, a small peak likely corresponding to

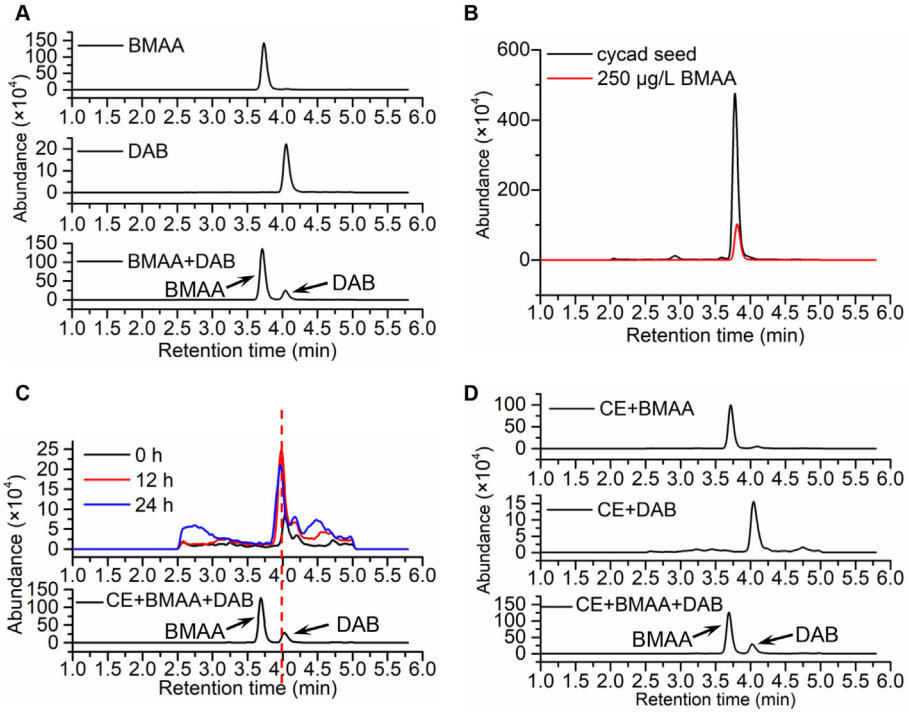

Fig. 2. Detection of BMAA and its isomer DAB by UPLC-MS/MS. (**A**) Chromatograms of DAB standard (100 μg/L) and/or BMAA standard (500 μg/L) analyzed by UPLC-MS/MS. (**B**) BMAA detection from cycad seed collected from Wuhan China. The red line shows the BMAA standard at 250 μg/L, and the black line shows the extract from cycad seeds. (**C**) Detection of BMAA and DAB in cell free extracts of *Nostoc* PCC 7120 following nitrogen deprivation. The upper chromatograms show the samples of *Nostoc* PCC 7120 cell free extracts after nitrogen limitation for 0 h (black line), 12 h (red line), 24 h (blue line), respectively. The lower chromatogram shows DAB standard (100 μg/L) and BMAA standard (500 μg/L). (**D**) Chromatograms of cell free extract (CE) of *Nostoc* PCC 7120 mixed with DAB standard or (and) BMAA standard. The concentrations of BMAA and DAB standards are the same as in (**A**).

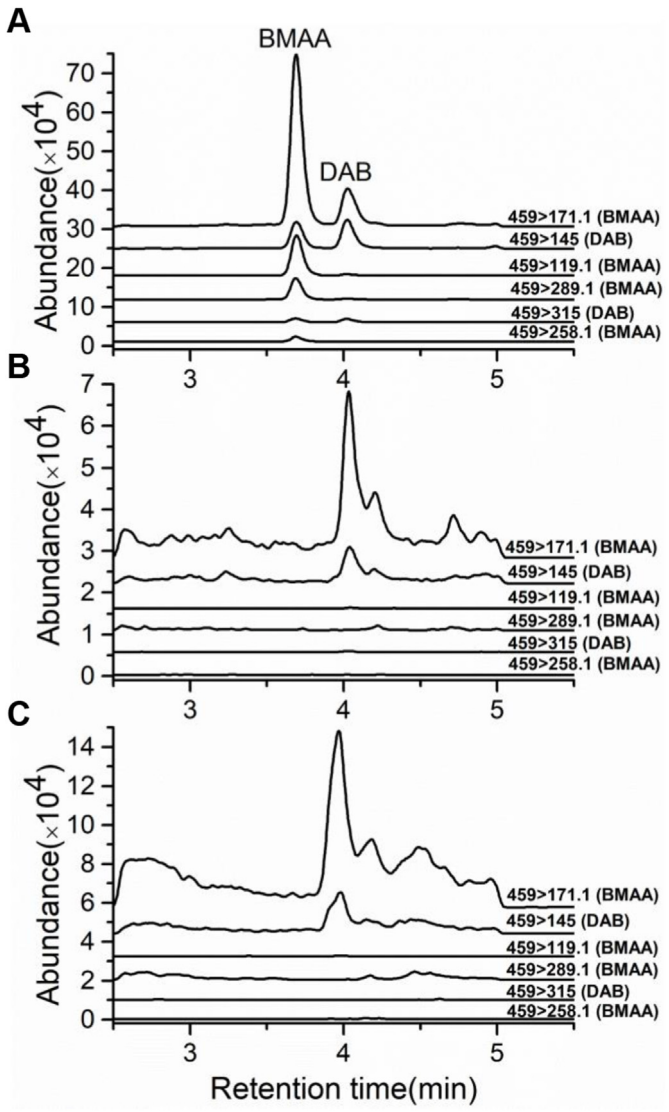

Fig. 3. Chromatograms combined with 6 different ions signals from AQC-UPLC-MS/MS analysis of standards and samples. (**A**) DAB standard (100 μg/L) and BMAA standard (500 μg/L). (**B**) The samples of *Nostoc* PCC 7120 cell free extracts after nitrogen limitation for 0 h. (**C**) The samples of *Nostoc* PCC 7120 cell free extracts after nitrogen limitation for 24 h.

BMAA was identified in the cell free extract obtained from a culture incubated in $BG11_0$ (Fig. 4B). To validate that the signal found in the $BG11_0$ culture could correspond to BMAA, we compared the 4 ions used to characterize BMAA by mass spectroscopy in this sample to those from BMAA and DAB standards (Fig. 4C and D). At least two ions characteristic of BMAA (Figs. 4C), 459 > 119.1 and 459 > 258.10, were completely missing from the sample prepared from cells with prolonged incubation in $BG11_0$ (Fig. 4D). These results demonstrated that BMAA was not present in the sample, and the signal with a similar retention time belonged to a different metabolite.

In a previous study, we found that BMAA was taken up via amino acid transport systems. Here, we further examined whether BMAA taken up into the cells from the growth medium could be metabolized. To do so, cells of *Nostoc* PCC 7120 were incubated with medium containing 50 μM BMAA for 20 min, washed three times and resuspended with BMAA-free medium. Cell free extracts were then prepared after 10, 30 and 60 min, and the amount of BMAA was determined. As shown in Fig. 5, the relative amount of BMAA detected in cell free extracts remained stable for up to 1 h after the removal of BMAA from the culture medium, indicating that BMAA, once in the cells, was poorly metabolized. These results suggest the absence of a specific detoxifying mechanism of BMAA in the cells.

3.4. Detection of BMAA in other cyanobacteria and bloom sample

The amount of BMAA reported in *Nostoc* PCC 7120 was relatively low compared to other cyanobacterial species (Cox et al., 2005). We extended our studies to include 29 species of cyanobacteria from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB) (Table 1). These strains included several species of *Nostoc* which were usually thought to produce high amounts of BMAA. This property is helpful for intracellular detection of BMAA. Surprisingly, the UPLC-MS/MS results showed that no BMAA was detected in any of these 29 cyanobacterial species, while the 2, 4-DAB was detected in some *Nostoc* strains, for example *Nostoc* sp. FACHB-1053 and *Nostoc* sp. FACHB-1155 (Fig. 6). Cyanobacteria maintained under laboratory conditions may lose some properties not essential for survival. Therefore, we sought to detect BMAA from natural cyanobacterial samples. For this purpose, we used 6 cyanobacterial samples collected at different times from blooms formed in two Chinese lakes, Lake Dianchi and Lake Taihu (Fig. 6, Table 2). Again, no BMAA was detected.

4. Discussion

The results of our studies call into question the capacity for BMAA biosynthesis in cyanobacteria. First, the UPLC-MS/MS method used in this study has a detection limit among the lowest so far reported. Secondly, the BMAA isomer, DAB, produced by a variety of microorganisms as a secondary metabolite, was detected, and was found to accumulate in response to nitrogen limitation (Fig. 2C). Thirdly, BMAA added to cell free extracts, or incubated with cell cultures, was easily detected and quantitated by our method (Wang et al., 2020). At the same time, we used a mutant which could no longer take up BMAA as a control, and found that when BMAA could not enter the cells, it could not be detected (Wang et al., 2020). These results, together with cycad seeds as a positive control, further validated our detection procedure. Finally, none of the 29 cyanobacteria strains of different genera from a culture collection could show a detectable level of BMAA. Note among the strains examined, *Nostoc* PCC 7120 and *Microcystis aeruginosa* PCC 7806 were listed as producers of BMAA, and the *Nostoc* strains used in our studies were closely related to those used in the previous reports (Cox et al., 2005). Cyanobacteria maintained under laboratory conditions may alter or even lose their ability to produce certain secondary metabolites. Therefore, we also included six cyanobacterial bloom samples from two different lakes. It is also worth noting that our experiments were performed in two independent laboratories with consistent results (CCZ's laboratory and Song's laboratory), one with expertise in genetic studies of cyanobacteria, and the other in cyanobacterial ecology and toxins.

Since the first report on the production of BMAA by cyanobacteria and the potential roles in the development of neurodegenerative diseases, considerable interests have been developed in the scientific community on the identification of BMAA from cyanobacterial samples. However, with time, the production of BMAA by cyanobacteria has become a controversial issue (Faassen et al., 2012; Faassen, 2014; Lage et al., 2016; Chernoff et al., 2017; Dunlop et al., 2021). The identification of BMAA has been reported in various species of cyanobacteria, including *Nostoc* PCC 7120 with a concentration determined at 32 μg/g DW for BMAA (Cox et al., 2005). Other studies failed to detect BMAA in cyanobacteria (Kubo et al., 2008; Rosen and Hellenas, 2008; Moura et al., 2009; Kruger et al., 2010; Li et al., 2012; Réveillon et al., 2014; Fan et al., 2015), and the detection methodology has been very often cited as one of the factors that explain such discrepancy (Faassen et al., 2012; Faassen, 2014; Lage et al., 2016; Manolidi et al., 2018; Bishop and Murch, 2019). We could detect exogenously added BMAA with a sensitivity comparable to those reported, yet still failed to detect endogenously produced BMAA.

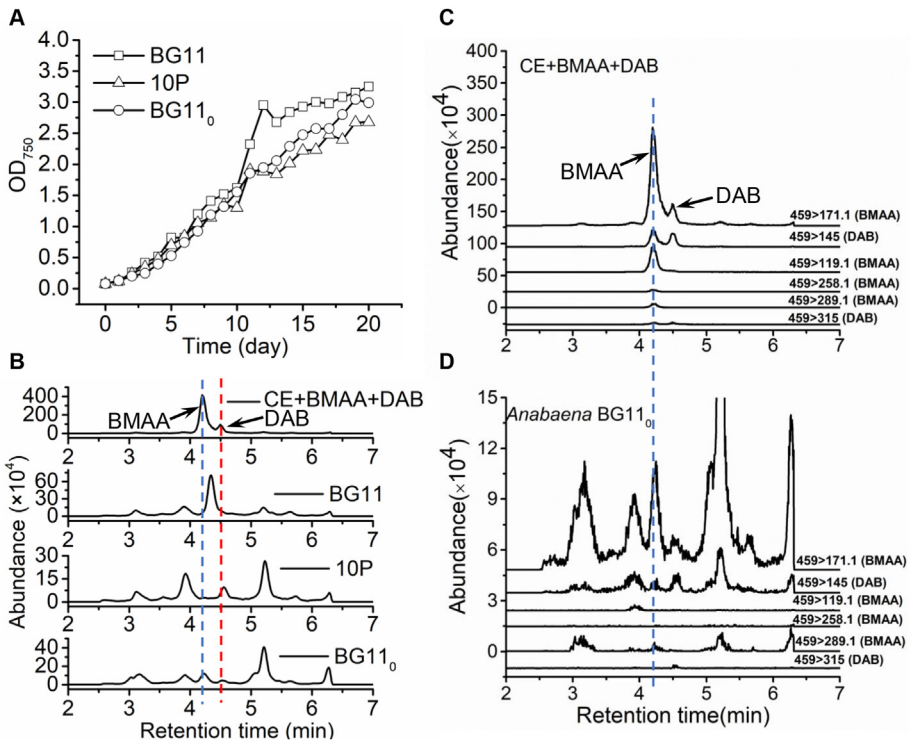

Fig. 4. BMAA detection from *Nostoc* PCC 7120 with prolonged incubation in BG11, BG11$_0$ and high phosphate (10P). (**A**) Growth curves of *Nostoc* PCC 7120 cultivated in BG11, BG11$_0$ and 10P. Data represents the mean values of two independent experiments. (**B**) Chromatograms of cell free extracts of *Nostoc* PCC 7120 cultured in BG11, BG11$_0$ and 10P for 20 days. (**C**, **D**) Chromatograms combined with 6 different fragment ion signals (459 > 119.1, 459 > 171.1, 459 > 258.1 and 459 > 289.1 are for BMAA, and 459 > 145 and 459 > 315 are for DAB) from AQC-UPLC-MS/MS analysis of standards (**C**) and cell free extract from *Nostoc* PCC 7120 cultured in BG11$_0$ for 20 days (**D**).

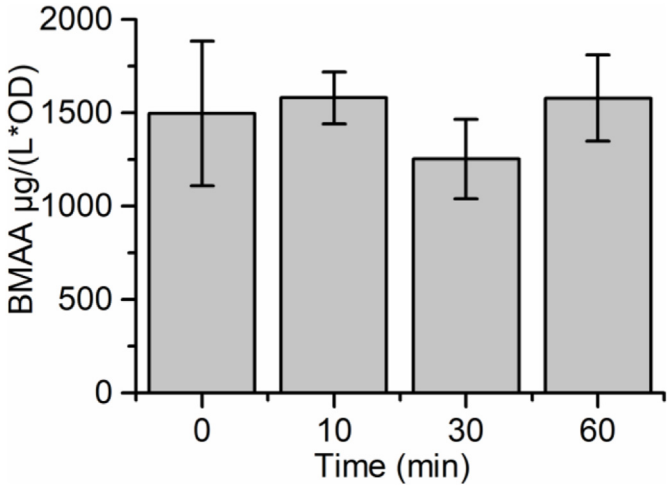

Fig. 5. The stability of BMAA in *Nostoc* PCC 7120 cells. Cells were incubated with BG11 containing 50 μM BMAA for 20 min, then washed three times with the same medium without BMAA, and finally resuspended in the same BMAA-free medium. After suspension, samples were taken at 10, 30 and 60 min. BMAA in the cells were then quantified by using UPLC-MS/MS (in gray column). Error bars denote standard deviations following triplicate experiments.

Our proposal is consistent with the fact that BMAA as an amino acid analog, is toxic to cyanobacteria such as *Synechococcus* sp. TAU-MAC 0499, *Nostoc* PCC 7120 and *Synechocystis* PCC 6803 as reported previously (Downing et al., 2012; Berntzon et al., 2013; Vergou et al., 2020). If these cyanobacteria could produce endogenous BMAA, it would be inhibitory to cell growth. Such an inhibitory effect, even a weak one, would make these strains much less competitive in the environment, unless the cells have an immunity to BMAA cytotoxicity. For example, cells could decrease BMAA toxicity by rapidly metabolizing or reexporting it from the cells. However, we found no evidence for the existence of such a mechanism, since BMAA remains quite stable in the cells at least over 1 h (Fig. 5). A previous study also indicated that BMAA remained stable in the cells, and only slowly declined over a long period of time (Downing and Downing, 2016), consistent with our results, suggesting the lack of an active mechanism for BMAA metabolism or export.

Bacteria produce a variety of amino acid analogs or intermediates during biosynthesis or metabolism, such as DAB (Li et al., 2023). It cannot be excluded that the chromatographic signal corresponding to BMAA could be derived from other isomers such as DAB. The base peak ions of BMAA and DAB from UPLC-MS/MS are the same (Fig. 3A), and if BMAA and DAB were not separated well from the column, it was hard to accurately identify them. Indeed, the increase in DAB following nitrogen step-down in *Nostoc* PCC 7120 (Fig. 2C) is similar to the pattern reported for BMAA under the same stress conditions (Downing et al., 2011; Yan et al., 2020). In other laboratories, they detected DAB in cyanobacteria but no BMAA (Rosen and Hellenas, 2008; Kruger et al., 2010; Li et al., 2012; Réveillon et al., 2014; Fan et al., 2015; Wang et al., 2021). Intriguingly, 20 days incubation under diazotrophic conditions led to the appearance of a signal, with a retention time similar to that of BMAA. However, with further investigation, the fragment ions did not support this signal being BMAA. Thus, the relative retention time may not be sufficient for the identification of BMAA. In future, the identification of BMAA should be carried out with a full characterization of its mass ions by mass chromatography. Many published studies on BMAA often lack the primary chromatographic data for the identification of BMAA signal, complicating the comparison of the results. Our findings are also consistent with a report, which showed that the analytical method based on the measurement of fluorescent derivative of BMAA used in the initial studies (Cox et al., 2003, 2005) was poorly selective (Faassen et al., 2012; Faassen, 2014). The data published by Cox et al. was based on a FLD (Fluorescence Detector) method (Cox et al., 2003, 2005), which may give false positive results. Our findings and the genetic controls based on amino acid transport mutant should help the community to clarify the situation.

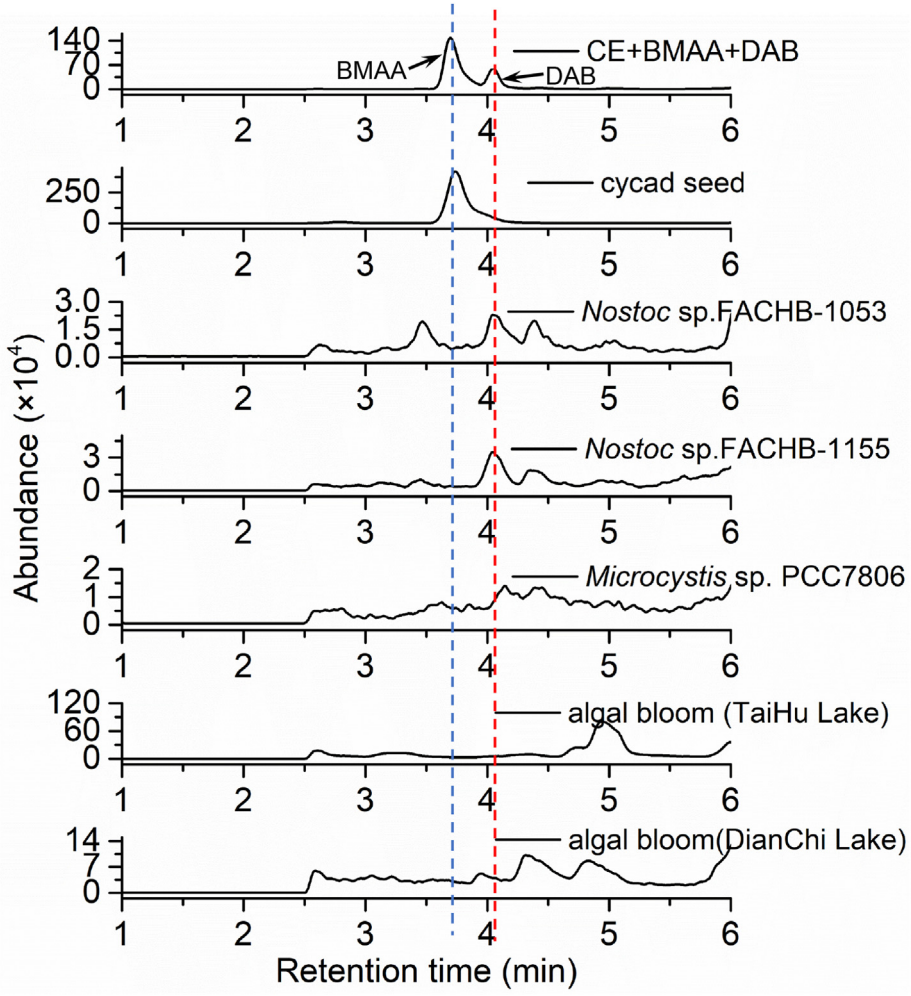

Fig. 6. Detection of BMAA and DAB from cyanobacteria UPLC-MS/MS. The chromatographs show the BMAA and DAB standard (mixed with *Nostoc* PCC 7120 cell free extract (CE)), and the extracts from cycad seeds, *Nostoc* sp, *Microcystis*, and algal bloom samples from Taihu Lake and Dianchi Lake in China. The blue dashed line shows the BMAA extension time. The red dashed line shows the DAB extension time.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Lirong Song and Cheng-Cai Zhang are editorial board members for *Water Biology and Security* and were not involved in the editorial review or the decision to publish this article.

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Author contributions

Zi-Qian Wang: Investigation, Writing- Original draft preparation, Writing- Reviewing and Editing. Suqin Wang: Investigation. Ju-Yuan Zhang: Methodology. Gui-Ming Lin: Investigation. Nanqin Gan: Conceptualization. Lirong Song: Conceptualization. Xiaoli Zeng: Conceptualization, Writing- Original draft preparation, Reviewing and Editing. Cheng-Cai Zhang: Conceptualization, Funding acquisition, Writing- Original draft preparation, Reviewing and Editing.

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