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RESEARCH ARTICLE

Effects of growth conditions on the production of neurotoxin 2,4-diaminobutyric acid (DAB) in *Microcystis aeruginosa* and its universal presence in diverse cyanobacteria isolated from freshwater in China

Hua Fan · Jiangbing Qiu · Lin Fan · Aifeng Li

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Abstract Neurotoxins *β*-*N*-methylamino-L-alanine (BMAA) and its isomer 2,4-diaminobutyric acid (DAB) have been reported previously in diverse strains of cyanobacteria. In this study, BMAA and DAB were analyzed for two strains of *Microcystis aeruginosa* incubated with four different levels of phosphate, nitrate, illumination, and temperature, respectively, in order to explore the effects of growth factors on toxin-producing ability of cyanobacteria. Both toxins were also screened in 17 cyanobacterial strains cultured with BG-11 medium and conventional illumination and temperature conditions, and in three field phytoplankton samples collected from different lakes in China. All samples were analyzed using a liquid chromatography-tandem quadrupole mass spectrometry (LC-MS/MS) system coupled with a hydrophilic interaction liquid chromatography (HILIC) column. Results showed that no BMAA was detected in any of the cyanobacterial strains grown under our laboratory culture conditions, or in any of the field samples. Production of DAB in *M. aeruginosa* was significantly enhanced by extreme concentrations of nutrient and physical factors. Various concentrations of DAB were also present in most cultured samples (13 of 17) of cyanobacteria and were not species specific. This is the first time to report the production of DAB in *M. aeruginosa* cultured under alterative conditions in laboratory. Occurrence of DAB in most of the strains examined here means that consideration should be given to the presence of this compound in freshwater environment in China.

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H. Fan · J. Qiu · L. Fan · A. Li
College of Environmental Science and Engineering, Ocean University of China, Qingdao 266100, China

A. Li (✉)
Key Laboratory of Marine Environment and Ecology, Ocean University of China, Ministry of Education, Qingdao 266100, China
e-mail: lafouc@ouc.edu.cn

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Introduction

The non-protein amino acid *β*-*N*-methylamino-L-alanine (BMAA) has been linked to the neurodegenerative disease amyotrophic lateral sclerosis-parkinsonism dementia complex (ALS-PDC) (Spencer et al. 1987; Banack and Cox 2003a; Monson et al. 2003). A diverse group of cyanobacteria has been reported to contain BMAA at a range of concentrations, suggesting that cyanobacterial blooms in freshwater resources could be a globally distributed risk to human health (Cox et al. 2005; Esterhuizen and Downing 2008; Metcalf et al. 2008). The accumulation and transfer of BMAA through the food chains have also been reported (Cox et al. 2003; Bidigare et al. 2009; Jonasson et al. 2010).

2,4-Diaminobutyric acid (DAB), as an isomer of BMAA, has previously been detected in diverse cyanobacterial samples (Rosén and Hellenäs 2008; Banack et al. 2010; Krüger et al. 2010; Richer et al. 2014; Metcalf et al. 2014). Typically, it has been identified as a diagnostic diamino acid in the cell wall peptidoglycan of 65 strains of actinomycetes (Groth et al. 1996; Suzuki et al. 1996). Within the flatpea (*Lathyrus sylvestris*), DAB was hypothesized to be responsible for the toxicity of this forage to some livestock (Shen et al. 1990). In addition to direct toxicity, DAB also significantly enhanced the toxicity of α-flupenthixol and pilocarpine inducing catalepsy of rats with intraperitoneal drug administration (Williams and Davies 1979). Since BMAA has been implicated as a pathogenic factor for the neurodegenerative disease ALS of humans, and BMAA and DAB frequently co-occur, it is possible that DAB also plays a role in disease pathogenesis.

So, the presence of DAB in cyanobacteria should be paid attention to in order to protect human health.

Extreme conditions have been known to alter the production of toxins in cyanobacteria. It has been proposed that the production of some cyanobacterial toxins is a stress response to unfavorable growth conditions (Rapala and Sivonen 1998; Hobson and Fallowfield 2003; Neilan et al. 2013). Reportedly, the production of BMAA in cyanobacteria was enhanced during the stress of nitrogen starvation (Downing et al. 2011). The concentration of DAB also increased in *L. sylvestris* as a result of drought condition (Shen et al. 1990). Therefore, alterative growth conditions should be considered in order to explore the production of toxins and further assess the potential risk of toxins to ecosystem.

The aim of this study was to explore the effects of growth conditions on the production of BMAA and DAB in *Microcystis aeruginosa*, a dominant species within cyanobacterial blooms in China. In combination with 17 cyanobacterial cultures originally isolated from Chinese freshwaters, as well as three field samples, the risk of BMAA and DAB in freshwater environments was examined. Different tissues of a cycad plant (*Cycas revoluta*) were analyzed to demonstrate the fitness for purpose of the liquid chromatography-tandem quadrupole mass spectrometry (LC-MS/MS) method for determination of BMAA in biological samples.

Materials and methods

Chemicals

BMAA (L-BMAA hydrochloride, B107, 10 mg) and DL-2,4-diaminobutyric acid dihydrochloride (DAB, D3758, 1 g) standards were purchased from Sigma-Aldrich. A mixed standard of BMAA (1.0 mg mL^{-1}) and DAB (1.0 mg mL^{-1}) was prepared in 2 mmol L^{-1} hydrochloric acid (HCl). The standard was diluted in 2 mmol L^{-1} HCl to prepare a series of working solutions for LC-MS/MS calibration (0.005, 0.05, 0.25, 0.5, 1.25, and 5.0 μg mL^{-1}). Methanol and acetonitrile purchased from Merck were high-performance liquid chromatography (HPLC) grade. HCl and ammonium hydroxide were purchased from Sinopharm Chemical Reagent. Formic acid (FA) was obtained from Sigma-Aldrich. Water used in these experiments was purified with a Milli-Q ultrapure water system (Millipore) to 18-MΩ quality or better.

Cyanobacteria cultured under alterative conditions

Microcystis spp. are dominant species of cyanobacterial blooms in freshwater environments of China during summer months. Two strains of *M. aeruginosa* were purchased from the freshwater algae collection in the Institute of Hydrobiology (FACHB), Chinese Academy of Sciences, and were recorded as "FACHB-905" and "FACHB-315" in this paper. The nutrient recipe of medium was based on BG-11 medium described by Rippka et al (1979). Four concentrations of phosphate ($K_2HPO_4{\cdot}3H_2O$) and nitrate ($NaNO_3$) along with four levels of physical factors illumination intensity (lx) and temperature (°C) were changed separately for every treatment based on the other three factors held to conventional levels (Table 1). Conventional levels of illumination intensity (lx), temperature (°C), $K_2HPO_4{\cdot}3H_2O$, and $NaNO_3$ were 3,000 lx, 20 °C, 0.175 mmol L^{-1}, and 17.6 mmol L^{-1}, respectively. The cyanobacterial strains are typically grown with these conditions in the FACHB. A total of 16 treatments were used to culture FACHB-905 and FACHB-315, with triplicate samples of each strain for each condition. One sample was used for cell counts to record growth status and to determine when to collect cells for toxin analysis, while cells from the remaining two samples were collected at the exponential and stable growth stages, respectively. All cultures were maintained under a 12-h light/12-h dark scheme in 300 mL of sterile medium in 500-mL borosilicate flasks.

Diverse cyanobacterial cultures and field samples

Seventeen cyanobacterial strains from ten genera were obtained from the FACHB and cultured under conventional conditions described above. They are common strains of cyanobacteria living in freshwater environments in China. Cultures were maintained in 150 mL of sterile BG-11 medium in 250-mL borosilicate flasks and collected after 30-day incubation which typically represented the stable growth stage. All cultures were maintained under a 12-h light/12-h dark scheme. Some species of cyanobacteria were filtered and collected by 0.45-μm membrane depending on their growth status, and the other cyanobacterial cultures were collected by centrifugation at 5,000×*g* for 10 min. Wet weights of these cultures were recorded. Three field samples of phytoplankton were collected from Lake Taihu, Lake Chaohu, and Lake Hongzehu in China, in July 2012. About 10 L of sampling water was filtered through the 0.45-μm membrane, and the wet weight of phytoplankton was recorded.

Extraction and SPE purification

All samples were stored at −20 °C before extraction. Cyanobacterial samples incubated with different concentrations of phosphate were extracted and analyzed in the Institute for Marine Biosciences, National Research Council (NRC), Canada, using the procedures described in our previous paper (Li et al. 2012). The other samples were extracted and analyzed in the Ocean University of China (OUC), China, using extraction and cleanup procedures with some minor modifications. These cyanobacterial cells were mixed with 6 mL of 75 % methanol solution (75/25 methanol/ water, *v*/*v*) and were

Table 1 Alterative levels of growth conditions for *Microcystis aeruginosa* in laboratory

Factors	Illumination intensity (lx)	Temperature (°C)	$K_2HPO_4 \cdot 3H_2O$ (mmol L^{-1})	$NaNO_3$ (mmol L^{-1})
Level 1	1,000	15	0.0175	0.00
Level 2	3,000[a]	20[a]	0.175[a]	1.76
Level 3	6,000	25	1.75	17.6[a]
Level 4	9,000	30	8.75	52.8

[a] Conventional growth conditions for this experiment

sonicated using a sonication probe for 15 min. The extraction solution was centrifuged at 8,000×*g* for 10 min, and the residue was extracted again in 3 mL of 75 % methanol solution and centrifuged again at 8,000×*g* for 10 min. The supernatants were combined, dried using N_2 (50 °C), and subsequently reconstituted in 1 mL of 20 mmol L^{-1} HCl as the free amino acid fraction. The protein pellet from the second extraction was transferred to a glass vial for hydrolysis in 6 mol L^{-1} HCl at 110 °C for 24 h. The solvent volume (mL) necessary to suspend the protein pellet was approximately two times the wet weight (g) of the cyanobacteria. Following hydrolysis, the solution was centrifuged at 8,000×*g* for 10 min and the supernatant was evaporated under N_2 (55 °C). This protein residue was reconstituted in 1 mL of 20 mmol L^{-1} HCl (0.5~5.6 g mL^{-1}) as protein-bound amino acid fraction. On to an Oasis cartridge (Waters, Oasis MCX, 3 cc, 60 mg), 0.8 mL of each free and protein-bound extract was loaded, and the eluant was dried under N_2 (50 °C) and then dissolved in 0.8 mL of 20 mmol L^{-1} HCl. All extracts were filtered through 0.22-μm nylon membrane before LC-MS/MS analysis.

Preparation of cycad tissues

A cycad plant (*C. revoluta*) was purchased from a flower market in Qingdao, China. Root, stem, and leaf tissues of this plant were separated and cleaned using pure water. The excess liquid was removed by blotting with paper towel. Portions (~1 g) of cycad tissues were placed in a mortar and ground with 5 mL of 75 % methanol solution (75/25 methanol/water, *v*/*v*). The extract was centrifuged at 8,000×*g* for 10 min. The supernatant was dried under N_2 (50 °C) and reconstituted in 1 mL of 20 mmol L^{-1} HCl. The protein residue was then hydrolyzed in 3 mL HCl (6 mol L^{-1}) at 110 °C for 24 h. The hydrolysis solution was dried under N_2 (55 °C) following centrifugation at 8,000×*g* for 10 min. The protein-bound extract was also reconstituted in 1 mL 20 mmol L^{-1} HCl. Using the solid-phase extraction (SPE) purification process applied above before analysis, 0.8 mL of free and protein-bound fractions was purified.

LC-MS/MS analysis

The cyanobacterial cultures grown under different phosphate concentrations were analyzed by an Agilent 1200 LC (Palo Alta) coupled with an AB-Sciex (Concord) API 4000 Qtrap mass spectrometer (Li et al. 2012). Other samples were analyzed by an Agilent 1290 HPLC (Palo Alta) coupled with an Agilent 6430 mass spectrometer with an electro-spray ionization source. A 5-μm TSKgel Amide-80® hydrophilic interaction liquid chromatography (HILIC) column (250 mm×2 mm internal diameter, Tosoh Bioscience LLC) was maintained at 40 °C to separate BMAA and DAB using a binary mobile phase system composed of water (solvent A) and 95 % acetonitrile (solvent B) each with 50 mmol L^{-1} FA. The gradient was linear from 90 to 60 % B over 15 min and was subsequently held for 4 min, decreased to 55 % B at 19.01 min, and held again for 7.99 min before re-equilibration. The flow rate was set at 350 μL min^{-1}, and an injection volume of 5 μL was used throughout. The MS was operated in positive ion mode with detection by the multiple reaction monitoring (MRM) mode to quantitate BMAA and DAB. Five transitions including 119>102, 119>101, 119>88, 119>56, and 119>44 were monitored at collision energies (CE) of 8, 5, 8, 15, and 20 V, respectively. The electro-spray voltage was set to 5,500 V with a source temperature of 450 °C. Nitrogen was used for the nebulizer and curtain gases. The *m*/*z* 119>102 and 119>101 transitions were used to quantitate BMAA and DAB, respectively. Signal intensity ratio between different MRM transitions was used as a supporting criterion for BMAA and DAB confirmation. Three low concentrations (0.4, 2, and 10 μg L^{-1}) of BMAA and DAB standards were spiked into one purified extract of FACHB-905, in order to determine the limit of detection (LOD) of LC-MS/MS method for both toxins.

Results and discussion

BMAA was detected in cycad

The LC-MS/MS method established on an Agilent 6430 mass spectrometer was used to analyze most of samples in the present study in OUC. Chromatograms of a mixed standard (Fig. 1a), a cyanobacterial extract (Fig. 1b), a field phytoplankton sample extract (Fig. 1c), and the cycad root extract (Fig. 1d) are shown. The chromatograms showed that BMAA and DAB could be separated well on the HILIC column used

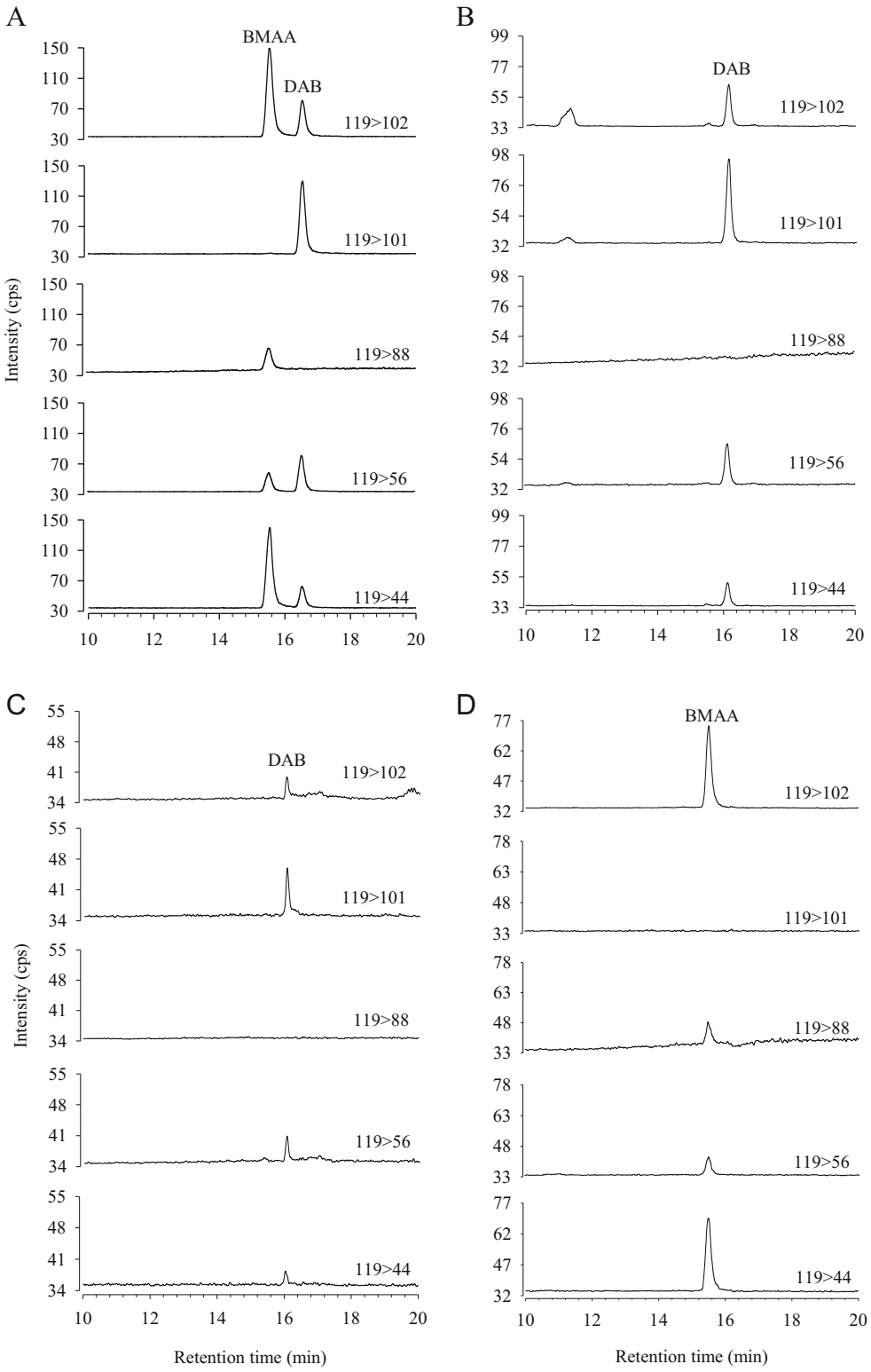

Fig. 1 LC-MS/MS chromatograms for BMAA and DAB in a mixed standard (0.05 μg mL^{-1}) (**a**), a laboratory culture of *Nostoc* sp. (**b**), a mixed plankton field sample collected from Lake Taihu (**c**), and an extract of cycad root (**d**). LC-MS/MS conditions as described in the "Materials and Methods" section using an Agilent 1290 HPLC coupled with an Agilent 6430 mass spectrometer

here. Different MRM transitions were used to identify BMAA and DAB, with the absence of response for BMAA in *m*/*z* 119>101 and DAB in *m*/*z* 119>88 as an important aspect in compound differentiation (Fig. 1). Peak area ratios for BMAA and DAB between the different MRM transitions were also an important criterion for analyte confirmation. Another isomer of BMAA, *N*-(2-aminoethyl) glycine (AEG), was also checked using this method. The three compounds were separated well on the HILIC column with AEG (17.14 min) eluting later than DAB (16.37 min). Several cyanobacterial

samples were examined for the presence of AEG, but none was identified.

Concentrations of BMAA and DAB in the root, stem, and leaf tissues are shown in Table 2. BMAA was detected in free form extracts of three different tissues of cycad without obvious difference. However, the concentration of BMAA in protein-bound root extracts was higher than that found in the stem or leaf. A detailed distribution of BMAA throughout different cycad tissues was not possible because only one plant sample was analyzed. The positive results of BMAA showed that the sample preparation procedures and LC-MS/MS method used here are effective for the determination of BMAA in cycads which are known to contain BMAA (Dossaji and Bell 1973; Schneider et al. 2002). However, almost no DAB was present in cycad tissues except for a trace amount of DAB detected in the protein-bound root extract. Trace amounts of DAB were also detected in several samples (3/18) of cycad (*Cycas micronesica*) tissues (Banack and Cox 2003b). It has not yet been established if cycads directly produce DAB or if it may be produced by the symbiotic cyanobacteria as shown in the case of BMAA (Cox et al. 2003).

LOD for BMAA as 2.0 pg was determined in one injection of 5-μL spiked sample with 0.4 μg L^{-1} standard when the signal to noise ratio (S/N) in at least three transitions used for qualitative diagnosis was above 3:1, and the LOD for DAB as 5.0 pg (S/N=3) was determined in one injection of 5-μL standard solution (5 μg L^{-1}). Therefore, the LC-MS/MS method used here is sensitive for BMAA and DAB compounds.

Production of DAB in *M. aeruginosa* strains cultured with alterative conditions

Growth curves of *M. aeruginosa* FACHB-315 and FACHB-905 cultured with alterative nutrient and physical factors are shown in the Fig. 2. No obvious differences occurred in the cultures with different levels of illumination and temperature compared with conventional physical conditions in this study. BMAA, either in the free or protein-bound form, was not detected in any of the cyanobacterial cultures grown under varying conditions as part of this work. However, different trace amounts of DAB were detected in both strains of *M. aeruginosa* (Fig. 3). In previous studies, DAB has also been reported in many species of cyanobacteria (Rosén and Hellenäs 2008; Krüger et al. 2010).

Table 2 Concentrations of BMAA measured in different tissues of cycad *Cycas revoluta*

Extraction	*Cycas revoluta* (μg g^{-1} wet weight)					
	Root		Stem		Leaf	
	BMAA	DAB	BMAA	DAB	BMAA	DAB
Free form	0.14	nd	0.18	nd	0.15	nd
Protein bound	0.13	0.0088	0.082	nd	0.018	nd

nd concentration was below the limit of detection (<LOD)

Growth rates of the FACHB-315 and FACHB-905 cultures were negatively affected by the highest phosphate concentration (8.75 mmol L^{-1}) when compared to other three lower concentrations (Fig. 2a, c). The cyanobacteria grew well with the three higher nitrate concentrations (1.76, 17.6, and 52.8 mmol L^{-1}), but growth slowed significantly in the absence of nitrate (no adding) (Fig. 2b, d). Until now, there has been limited information in the literature regarding the potential for cyanobacteria to produce BMAA and DAB under various culture conditions. It has previously been reported that BMAA production is stimulated under nitrogen limited conditions (Downing et al. 2011). The highest concentration of DAB was produced by the FACHB-315 strain cultured in the absence of nitrate in this study, which demonstrated that the production of DAB also followed this stress rule. Concentrations of DAB significantly increased in both strains of *M. aeruginosa* with enhancing concentration of phosphate. This suggests that a low nitrate to phosphate (N/P) ratio stimulates DAB production. Interestingly, the highest concentration of DAB was found in the *M. aeruginosa* strains cultured with the phosphate level of 8.75 mmol L^{-1}, even though cellular reproduction was the lowest under this particular growth condition (Fig. 2). This phenomenon showed that the production of DAB may be enhanced under adverse growth conditions, as has previously been reported for microcystins (Wang et al. 2002; Neilan et al. 2013). The production of microcystins was also assessed in these cultures, and it was found that the concentrations of microcystin (MC)-LR and [Dha^7] MC-LR obviously increased in the cultures under the highest concentration of phosphate (8.75 mmol L^{-1}) applied in this study, even in the non-toxic strain (FACHB-315) for microcystins when growing under conventional conditions (data not shown). These results indicate that the production of both DAB and microcystins is stimulated by the high phosphate concentration in the culture medium. It should be mentioned that the cultures under different phosphate conditions were determined by an LC-MS/MS system used in our previous study (Li et al. 2012). The other cyanobacterial cultures used in this study (nitrate, temperature, illumination) were analyzed using the LC-MS/MS method developed in OUC. It is reasonable to compare the concentrations of DAB in the experimental group with four different phosphate levels. Generally, the highest concentrations of DAB were present in cultures grown under the highest light intensity (9,000 lx), indicating that extreme illumination intensity increases DAB production. In the experimental group of different temperatures, the highest concentrations of DAB occurred in the cultures under the highest temperature (30 °C) at the stable

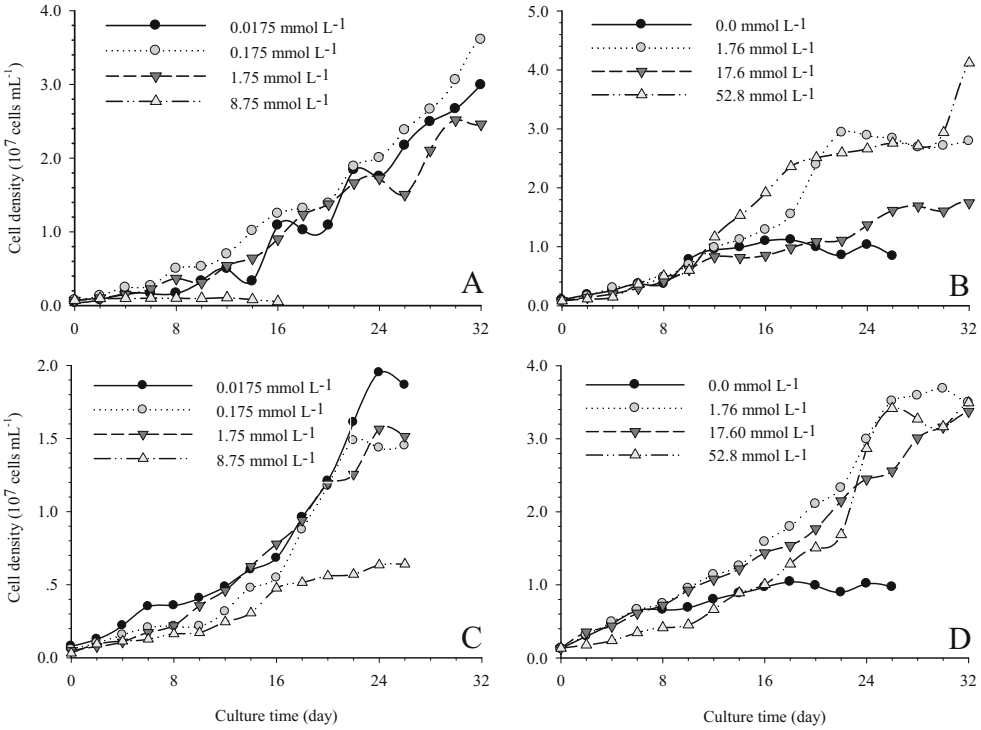

Fig. 2 Growth curves of *Microcystis aeruginosa* FACHB-315 (**a**, **b**) and FACHB-905 (**c**, **d**) cultured with altered BG-11 medium with different levels of phosphate concentration (**a**, **c**) and nitrate concentration (**b**, **d**)

growth stage. In total, the temperature factor had a limited influence on the observed DAB concentrations. It should be noted that the concentrations of DAB measured here were generally low; therefore, it is difficult to draw any definitive conclusions on the effects of the variations investigated. Significantly, the alterative environmental conditions had no influence of BMAA production for both particular strains, with concentrations of BMAA < LOD in all samples.

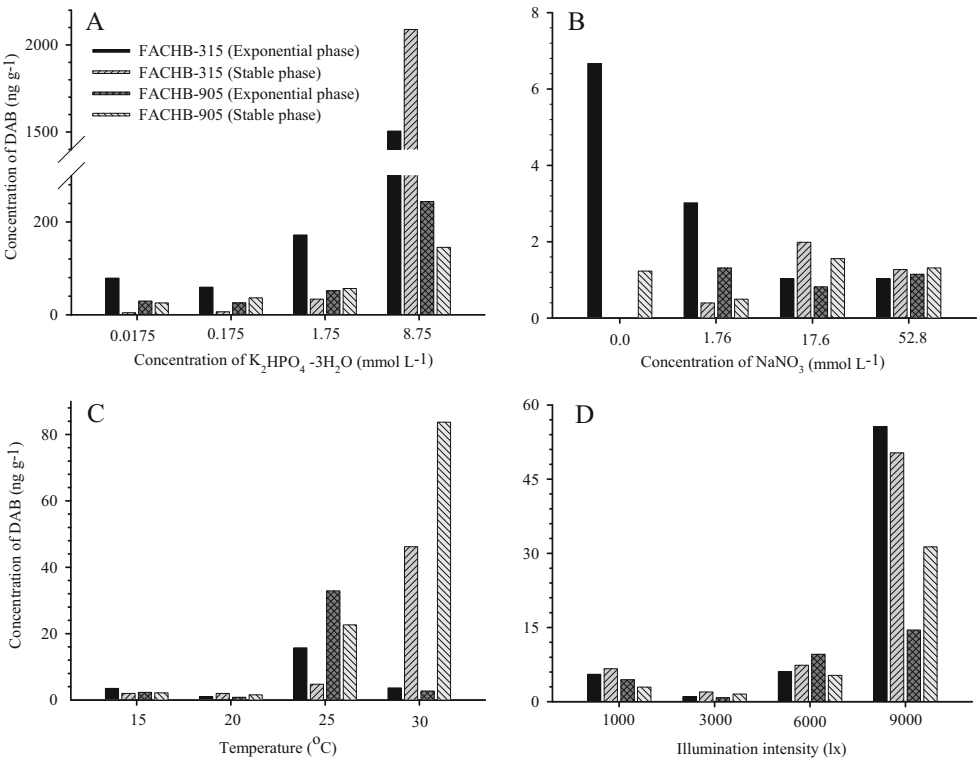

Fig. 3 Total concentrations of DAB in free and protein-bound fractions of *Microcystis aeruginosa* FACHB-315 and FACHB-905 cultured with different levels of phosphate (**a**), nitrate (**b**), temperature (**c**), and illumination intensity (**d**) in exponential and stable growth phases. The concentrations of DAB in cyanobacterial cells cultured under different concentrations of phosphate were analyzed using the API 4000 Qtrap mass spectrometer in NRC, Canada

Table 3 Concentrations of DAB detected in diverse cyanobacteria isolated from Chinese freshwater and cultured with conventional conditions

Genera	Code	Origin	Collection	DAB (ng g^{-1} wet weight)	
				Free	Bound
Nostoc sp.	FACHB-106	Changsha	Filtration	0.71	nd
	FACHB-973	Lake Donghu,	Centrifugation	3.18	nd
Calothrix sp.	FACHB-154	Changde	Filtration	2.07	nd
	FACHB-167	Wuchang	Filtration	12.22	2.74
Scytonema sp.	FACHB-626	China	Filtration	0.36	nd
Lyngbya maiussula Harv	FACHB-866	Chengdu	Filtration	0.92	nd
Lyngbya cryptovaginatus	FACHB-890	China	Centrifugation	5.78	nd
Synechococcus sp.	FACHB-1061	Changjiang River	Centrifugation	15.69	nd
Anabaena sp.	FACHB-1091	Lake Donghu	Centrifugation	nd	nd
	FACHB-1180	Lake Chaohu	Centrifugation	5.63	nd
	FACHB-1263	Dianchi	Centrifugation	nd	nd
Planktothrix agardhii	FACHB-1243	Lake Chaohu	Centrifugation	0.71	nd
	FACHB-1261	Lake Taihu	Centrifugation	1.50	nd
Aphanizomenon flos-aquae	FACHB-1249	Lake Chaohu	Filtration	1.30	nd
	FACHB-1290	Dianchi	Centrifugation	0.81	nd
	FACHB-1171	Lake Taihu	Centrifugation	nd	nd
Plectonema phormidiodes	FACHB-200	Jiangsu	Centrifugation	nd	nd

nd concentration was below the limit of detection (<LOD)

Presence of DAB in diverse cyanobacteria and in field phytoplankton samples

Seventeen strains of cyanobacteria isolated from freshwater environments of China and sourced from the Chinese culture collection were grown under conventional conditions (20 °C, 3,000 lx, 0.175 mmol L^{-1} $K_2HPO_4{\cdot}3H_2O$, 17.6 mmol L^{-1} $NaNO_3$). Some of these cyanobacteria are common strains known to cause blooms in Chinese freshwater environments (Chen et al. 2003). No BMAA was found in any strains, but mainly free form (non-protein bound) of DAB was detected in 13 of 17 strains of cyanobacteria investigated here (Table 3). The highest concentration of DAB was detected as 15.69 ng g^{-1} wet weight in *Synechococcus* sp. (FACHB-1061) isolated from Yangtze River. Concentrations of DAB fluctuated significantly between these different cyanobacteria, even for strains belonging to the same genus. Protein-bound DAB was only found in one sample (2.74 ng g^{-1} wet weight), a strain of *Calothrix* sp. (FACHB-167) isolated from Wuchang, China.

Field samples of phytoplankton were collected from three lakes of importance as drinking water supplies in China. BMAA was not detected (<LOD) in any of the field samples taken, while DAB was detected in all three, with the highest concentration of 3.82 ng g^{-1} in the sample collected from Lake Hongzehu (Table 4). Cyanobacterial blooms frequently occurred in Lake Taihu and Lake Chaohu since the 1980s with the dominate cyanobacterial species being *Microcystis* spp. (Chen et al. 2003). Cyanobacterial blooms were forming in Lake Taihu and Lake Chaohu at the time of sampling for this study while no obvious bloom was present in Lake Hongzehu. The fluctuating concentrations of DAB in the cyanobacteria indicated that the production of DAB was not species specific. As well as a considerable concentration variation of BMAA ranged in diverse cyanobacteria in previous studies (Cox et al. 2005). Therefore, it is difficult to monitor and assess the risk of BMAA and DAB in cyanobacteria growing in freshwater environments. Presently, an average BMAA concentration of 4.12 μg g^{-1} dry weight was reported in cyanobacteria sampled

Table 4 Concentrations of DAB in field plankton samples collected from Chinese lakes

Lakes	Coordinates	Dominant genus	DAB (ng g^{-1} wet weight)	
			Free	Bound
Lake Taihu	31° 20′ N, 120° 20′ E	*Microcystis* spp.	1.46	nd
Lake Chaohu	31° 15′ N, 117° 30′ E	*Microcystis* spp.	0.37	nd
Lake Hongzehu	33° 20′ N, 118° 55′ E	*Microcystis* spp. *Anabaena flos-aquae*	3.82	nd

nd concentration was below the limit of detection (<LOD)

from the Gonghu Bay in Lake Taihu, China, using AQC derivatization prior to LC-MS/MS analysis (Jiao et al. 2014). BMAA has previously been reported as not detectable cyanobacterial samples from prior studies using a HILIC column rather than derivatization and a reverse-phase C18 column (Rosén and Hellenäs 2008; Krüger et al. 2010; Li et al. 2010). There is still a discrepancy between results for BMAA in cyanobacteria obtained by different analysis procedures. It is urgent to resolve this mysterious phenomenon in future works.

In addition to certain coryneform bacteria (Collins and Jones 1980; Groth et al. 1996; Sasaki et al. 1998), flatpea (*L. sylvestris*) (Foster et al. 1987), and cycad (Banack and Cox 2003b) as well as cyanobacteria containing neurotoxin DAB, it was also detected in bovine brain (3 μg kg^{-1} wet weight) (Nakajima et al. 1967) and Lesser Flamingo (*Phoeniconaias minor*) feathers (3.5–8.5 μg g^{-1} dry weight) sampled from lakes (Metcalf et al. 2013). The transfer and biomagnification of DAB along trophic food chains are still absent in freshwater environments. It is hypothesized that DAB does not like BMAA being easily embedded into the proteins of plants and animals. Only one sample of cultured cyanobacteria was identified as with trace amount of DAB in protein-bound fraction in this study, which is also supporting this hypothesis. Therefore, it is uncertain if the universal trace amounts of DAB within cyanobacteria are linked with the life quality of organisms living in freshwater environments, even with human health. More works should be carried out to investigate this neurotoxic amino acid in different organism samples collected from Chinese freshwater environments in order to further estimate its risk for ecosystem safety.

Summary

An effort was made here to explore the effects of growth conditions on the production of neurotoxins BMAA and DAB in typical strains of cyanobacteria from Chinese freshwater environments. Production of DAB in both strains of *M. aeruginosa* was significantly affected by extreme levels of phosphate and nitrate in medium, and the highest illumination intensity and temperature. DAB was present in diverse cyanobacteria isolated from Chinese freshwater environments. However, the production of BMAA was not stimulated by alterative conditions in the *M. aeruginosa* cultures and no BMAA was detected (<LOD) in any cyanobacteria and field samples studied here. Occurrence of DAB in most of the strains analyzed, either from laboratory cultures or from field samples, means that consideration should be given to the presence of this neurotoxic substance in freshwater resources. There appears to a minimal risk associated with BMAA being present in cyanobacteria prevalent in Chinese waters.

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Conflict of interest The authors declare that there is no conflict of interest.

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