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SINGLE-LABORATORY VALIDATION AND DETERMINATION OF TOTAL β-N-METHYLAMINOALANINE IN CYANOBACTERIA FROM AOTEAROA NEW ZEALAND

SINGLE-LABORATORY VALIDATION AND DETERMINATION OF TOTAL β-N-METHYLAMINOALANINE IN CYANOBACTERIA FROM AOTEAROA NEW ZEALAND

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EXECUTIVE SUMMARY

β-N-methylaminoalanine (BMAA) is a non-proteinogenic amino acid reported to be produced by cyanobacteria. Because of its possible links with neurodegenerative disorders such as amyotrophic lateral sclerosis and Parkinsonism–dementia complex, BMAA has generated growing interest over the past 20 years. However, its presence in cyanobacteria is still debated due to inconsistencies in the literature. To evaluate whether BMAA occurs in cyanobacteria from Aotearoa New Zealand, a direct quantitation method for 'total BMAA' was developed and validated.

Acid hydrolysis at high temperatures was used to release BMAA bound in cyanobacterial proteins / peptides; however, this resulted in extreme interference from proteinogenic amino acids (particularly arginine). The use of a cation exchange clean-up step minimised this interference and, with tuning of the mass spectrometric acquisition parameters, acceptable sensitivity was achieved.

The resulting hydrophilic interaction chromatography-tandem mass spectrometry (HILIC-MS/MS) method was validated on the criteria of selectivity, linearity, sensitivity, short-term precision, intermediate precision and accuracy in two freeze-dried cyanobacteria samples fortified with known levels of BMAA. A balance between sensitivity and selectivity for isobaric compounds (i.e. structurally related isomers of BMAA) was achieved through the selection and tuning of multiple-reaction monitoring transitions. Using two additional cyanobacterial matrices, the limits of detection and quantitation were estimated to be 93–247 µg/kg and 310–950 µg/kg, respectively. Accuracy assessments at fortification levels of 200 µg/kg, 500 µg/kg and 1,000 µg/kg demonstrated 95–125% recovery, and at fortification levels ≥ 500 µg/kg recovery was very good (95–106%). The limit of reporting was set at 300 µg/kg, acknowledging that the measurement accuracy from 300 µg/kg to 500 µg/kg could be impaired. The method precision was determined in two cyanobacterial matrices by evaluating the relative standard deviation (RSD) for replicate measurements of fortified cyanobacteria samples. In one of the validation samples, the short-term precision was 8.2% RSD_r and the intermediate precision was 9.4% RSD_R, but precision was poorer in the second validation sample, at 20.7% RSD_r and 19.9% RSD_R.

The total BMAA method was applied to 34 cyanobacteria samples from Aotearoa New Zealand. These samples included 20 different cyanobacterial genera and covered a range of morphologies and growth strategies. BMAA was detected in two cyanobacteria samples, which were both *Planktothrix* sp. The concentrations observed varied between the two *Planktothrix* sp. strains tested, where 100 µg/kg was observed in CAWBG-59 and 2,600 µg/kg was observed in CAWBG-35. The low rate of detection (only 6% of the samples tested were positive) observed in the current study corroborates similar findings from other studies investigating BMAA in cyanobacteria using direct analysis by HILIC-MS/MS.

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1. INTRODUCTION

β-N-methylaminoalanine (BMAA) is a non-proteinogenic amino acid that has been reported to be produced by cyanobacteria. BMAA is considered a possible cause of neurodegenerative disorders such as amyotrophic lateral sclerosis and Parkinsonism–dementia complex (ALS-PDC). The connection was first made among the Chamorro people of Guam, who have high rates of ALS-PDC. This was linked to the BMAA produced by cyanobacteria (*Nostoc* sp.) growing as a symbiont on cycad trees and biomagnifying through the food chain. In this instance, the Chamorro people ate fruit bats that feed on cycad seeds containing BMAA (Cox and Sacks 2002; Cox et al. 2003). The potential link between cyanobacteria and neurodegenerative disorders is also supported by epidemiological studies that have identified higher rates of these disorders in people living close to, or frequently visiting, lakes with cyanobacterial blooms (Sienko et al. 1990; Caller et al. 2009; Caller et al. 2012; Andrew et al. 2017; Fiore et al. 2020).

These observations have prompted research on the analysis of BMAA and its occurrence in cyanobacteria, microalgae and aquatic foodstuffs. However, analysis methods that derivatise BMAA (e.g. using chloroformate or AQC; 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate) appear to lead to detection of false positives (Chorus and Welker 2021; van Ginkel et al. 2022). Direct separation and measurement of BMAA using hydrophilic interaction chromatography coupled with tandem mass spectrometry detection (HILIC-MS/MS) is currently the most robust method for specific measurement of BMAA. This approach avoids interference from structurally related compounds (Figure 1) that can affect measurements. These compounds include:

- 2,4-diaminobutyric acid (2,4-DAB)
- 3,4-diaminobutyric acid (3,4-DAB)
- N-(2-aminoethyl)-glycine (AEG)
- β-amino-N-methylalanine (BAMA).

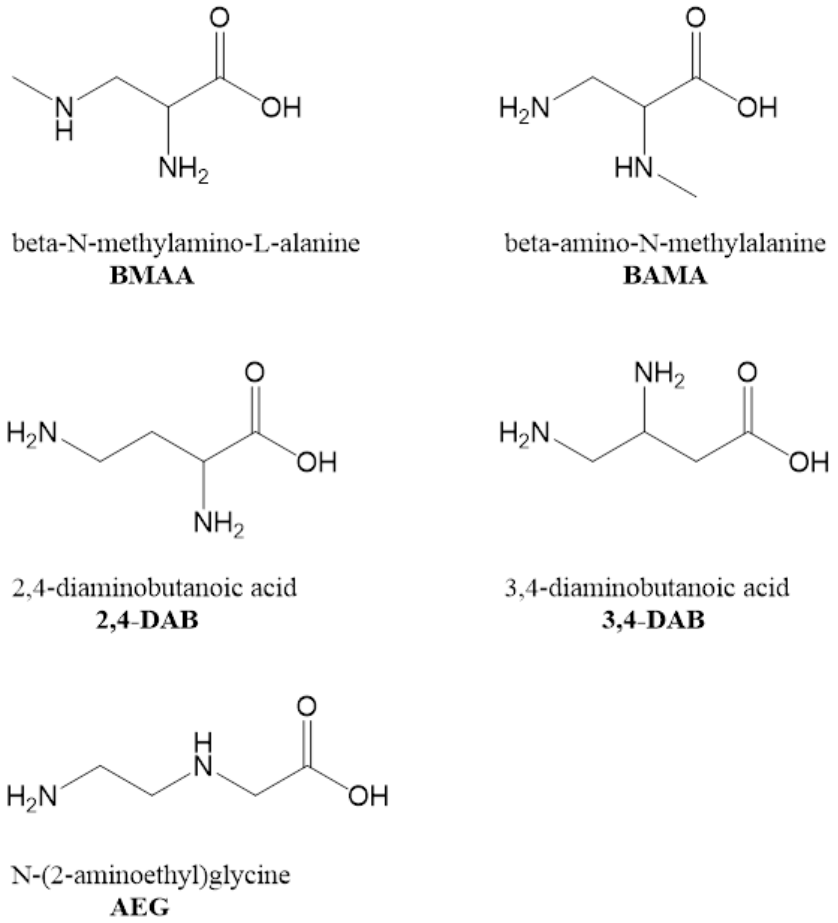

Figure 1. Structures of β-N-methylaminoalanine (BMAA) and structural isomers that can interfere in its analytical detection.

BMAA occurs in three different forms: free, soluble-bound and insoluble-bound (Faassen et al. 2016). Free BMAA is present as an amino acid in solution. Soluble-bound BMAA is incorporated into hydrophilic proteins / peptides that are in solution. Insoluble-bound BMAA is incorporated into hydrophobic proteins that are not in solution under normal conditions. Total BMAA is a combination of all three forms: free BMAA, soluble-bound BMAA and insoluble-bound BMAA.

In Cawthron Report 3796, we developed and validated a HILIC-MS/MS method to measure free BMAA in cyanobacteria (van Ginkel et al. 2022). In the current study, this method was adapted for the measurement of total BMAA. This involved the development of a hydrolysis method to liberate bound BMAA, removal of interfering amino acids through a solid-phase extraction (SPE) clean-up and adjustment of the previously developed HILIC-MS/MS method to achieve acceptable levels of sensitivity. The total BMAA method was validated on the criteria of selectivity, linearity, sensitivity, short-term precision, intermediate precision and accuracy in two cyanobacteria samples. It was then applied to the analysis of two environmental

cyanobacteria samples from Aotearoa New Zealand and 32 cultured cyanobacteria strains that have been isolated from lakes and rivers around Aotearoa New Zealand.

2. METHODS

2.1. Cyanobacteria samples

2.1.1. Strain selection and culturing

Cyanobacteria strains sourced from the Cawthron Institute Culture Collection of Microalgae (CICCM; *n* = 32; Rhodes et al. 2016) were grown in MLA (Bolch and Blackburn 1996) or F/2 media (Guillard and Ryther 1962). The cyanobacterial strains were selected to cover a range of different genera, morphologies and growth strategies (see Appendix 1 for details).

A sub-sample of approximately 5 mL of each cyanobacterial strain was aliquoted into a sterile 400 mL glass flask with freshly made MLA or F/2 media. The flasks were placed in a controlled temperature and light room at 17 °C (± 1 °C) with a 12 h / 12 h light / dark cycle. After several days, the strains were transferred to one of five different conditions to enable optimal growth (Table 1). The strains were monitored biweekly for growth until ready for harvesting (see Section 2.1.2). The growth method used for each strain is provided in Appendix 1.

Table 1. Growth methods used for cyanobacteria culturing (see Appendix 1 for a list of each strain and assigned growth method).

Growth method	Vessel	Temperature (°C)	Light / dark cycle	Light intensity (µmol/m²/s)
1	Glass flask	17 (± 1)	12 h / 12 h	27–37
2	Glass flask	17 (± 1)	12 h / 12 h	1 ± 0.5
3	Glass flask with aeration	17 (± 1)	12 h / 12 h	27–37
4	Blue 14 L plastic carboy	17 (± 1)	12 h / 12 h	25–37
5	Flat bags	17 (± 1)	24 h light	27–37

2.1.2. Harvesting cultured cyanobacteria

The harvesting method used for each strain is provided in Appendix 1.

Harvest method 1 – centrifuging (most strains)

When possible, strains grown in flat bags or 1 L culturing flasks were centrifuged (5,000 × *g*, 20 min; 20 °C) and the supernatant discarded, except for approximately 5 mL, which was retained to resuspend the pellet. This remaining medium with concentrated algae was transferred to a pre-weighed 50 mL polypropylene tube, which was centrifuged (3,000 × *g*, 10 min; 20 °C). The supernatant was discarded, and the remaining pellet stored at −20 °C until freeze-drying.

Harvest method 2 – pipetting (coarse filamentous strains and spongy balls)

Strains that grew as fibrous long filaments or spongy balls (see Figure 2 for examples) did not form dense pellets when centrifuged. For these strains, excess media was carefully decanted. A pipette was used to collect and transfer cyanobacteria to pre-weighed 50 mL polypropylene tubes, which were stored at −20 °C until freeze-drying.

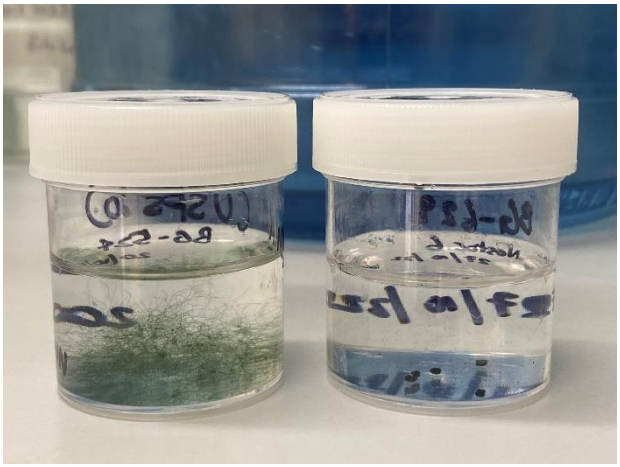

Figure 2. Examples of cyanobacterial strains that would not form pellets with centrifuging.

Harvest method 3 – pipetting (buoyant strains)

Some strains were highly buoyant and did not form a pellet after centrifuging. Glass culturing flasks containing these cyanobacterial strains were filled with MLA to the neck of the flask and left undisturbed for approximately 1 h. A pipette was used to collect the cyanobacteria floating on the surface. These were transferred to pre-weighed 50 mL polypropylene tubes, which were stored at −20 °C until freeze-drying.

Harvest method 4 – sieving (large colonial strains)

To harvest colonial cyanobacteria grown in large volumes (i.e. in 14 L carboys using growth method 4), nylon netting (20 µm; Particle Solutionz Ltd) was placed over a strainer on top of a bucket. The contents of the 14 L carboy were poured into the nylon netting, capturing the cyanobacteria colonies. Using a Pasteur pipette, cyanobacterial material was collected into pre-weighed 50 mL polypropylene tubes. The cyanobacteria were centrifuged (3,000 × *g*, 5 min; 20 °C) and the supernatant was discarded. The tubes were stored at −20 °C until freeze-drying.

2.1.3. Freeze-drying cultured cyanobacteria

All samples were stored at −20 °C for ≥ 12 h before starting the freeze-drying process. Prior to freeze-drying, tube lids were removed and replaced with a folded Kimwipe sheet secured with a rubber band. Samples were freeze-dried at 1.51 mbar vacuum

for a minimum of 24 h in a Christ 1-16 LSC-plus freeze dryer. The dried samples were crushed using a sterile spatula until a fine powder was formed.

2.1.4. Other cyanobacteria samples

- ALG-06: environmental benthic cyanobacterial mat material dominated by *Microcoleus autumnalis*. Collected from Te Awa Kairangi / Hutt River (Wellington region) on 31 March 2016, freeze-dried, crushed and stored at −20 °C.
- Spirulina: *Arthrospira platensis* commercially grown as a health supplement (Tahi™ Artisan Spirulina; 23 December 2019 batch).

2.2. Analytical methodology and validation

2.2.1. Standards

All primary standards and reference materials were obtained from the National Research Council Canada (Table 2). Working standards were prepared by diluting the primary standard to the required concentration using 2 mM hydrochloric acid (HCl).

Table 2. Primary standards used in this study (source: National Research Council Canada).

Reference material	Concentration (µg/mL)
BMAA	10
BAMA	690
2,4-DAB	650
3,4-DAB	690
AEG	960
$^{13}C^{15}N_2$-BMAA	26

2.2.2. Validation samples

Two samples of freeze-dried cyanobacteria (see Section 2.1.4) were used to determine accuracy and precision for the validation study:

- ALG-06: *Microcoleus autumnalis*-dominated benthic mats
- Spirulina: *Arthrospira platensis* commercially grown as a health supplement.

For the determination of the theoretical limit of detection (LoD) and limit of quantitation (LoQ), the two samples mentioned above were used in addition to two cultured cyanobacteria samples (see Section 2.1.1):

- *Oscillatoria* sp. CAWBG-104
- *Leptolyngbya* sp. CAWBG-114.

Insufficient biomass was available to include these cultured cyanobacteria samples in all the validation experiments.

2.2.3. Sample preparation

Cyanobacteria samples were weighed into 2 mL amber ampoules. Fortification spike (BMAA; when used) and internal standard ($^{13}C^{15}N_2$-BMAA; all samples) were added as per Table 3, then made to a final volume of 1 mL with 6 M of HCl (i.e. the volume of HCl added was reduced by the volume of spike and internal standard added). The ampoules were flame-sealed and heated to 115 °C for 15 h, with the low pH liberating bound BMAA. After cooling to room temperature, the contents were transferred to micro-centrifuge tubes and centrifuged (17,000 × *g*, 5 min). The supernatant was transferred to a 15 mL polypropylene tube and evaporated to dryness at 60 °C under a stream of nitrogen. The dried residues were resuspended in 0.5 mL of deionised water and centrifuged (4,000 × *g*, 5 min).

Table 3. Volumes and concentrations of internal and fortification standards.

Fortification level (µg/kg)	Sample weight (mg)	Spike added (µL of 1 µg/mL)	Internal std (µL of 1 µg/mL)	Dilution factor
0	25	0	25	100
200	25	5.0	25	100
500	25	12.5	25	100
1,000	25	25.0	25	100

A volume of each supernatant (200 µL) was loaded onto a 200 mg Strata-X-C (strong cation exchange) SPE cartridge. No pre-conditioning of the SPE cartridge was required. The SPE cartridges were eluted with 3 × 1 mL of 0.2 M phosphate buffer at pH 7. Fraction 2 contained the vast majority of BMAA and the internal standard, and these were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS; see Appendix 1 for instrument parameters). The presence of the internal standard $^{13}C^{15}N_2$-BMAA was used to compensate for any losses from the sample preparation, including the SPE clean-up.

2.2.4. Validation outline

To assess the performance of the total BMAA method, it was tested for selectivity, linearity, sensitivity, accuracy, short-term precision and intermediate precision. Each parameter was investigated in line with the validation experiments previously undertaken for the measurement of free BMAA documented in Cawthron Report 3796 (van Ginkel et al. 2022).

Selectivity

- Differentiation of BMAA, $^{13}C^{15}N_2$-BMAA (internal standard) and interfering BMAA isomers was achieved through chromatographic and mass spectrometric methods.
- Retention times and confirmation ratios of standards and spiked samples were compared to establish specificity.

Linearity

- A dilution series of a BMAA standard was prepared incorporating an internal standard to establish the linear range for the LC-MS/MS method.

Sensitivity

- The theoretical LoD and LoQ for freeze-dried cyanobacteria samples were determined from 16 or eight repeat analyses of four different samples, all spiked with BMAA at a low level.
- The LoD was calculated as 2.6 times the standard deviation of the 16-fold low-level repeat analyses and 3 times the standard deviation of the eightfold repeat analysis. The LoQ was calculated as 10 times the standard deviation.

Precision

- Short-term and intermediate precision were evaluated in four replicates of cyanobacteria samples fortified with 500 µg/kg of BMAA analysed within a single day and three repeat batches analysed over three subsequent days.
- The within-day (RSD_r) and inter-day (RSD_R) precision was approximated using calculations described by the International Organization for Standardization (2019).

Accuracy

- Interference (enhancement or suppression) from non-target matrix components was controlled using a stable isotope labelled internal standard.
- Two cyanobacterial samples were spiked at 200 µg/kg, 500 µg/kg and 1,000 µg/kg of BMAA and percentage recovery evaluated.
- Four replicates were prepared for the 500 µg/kg sample, while single samples were prepared for the other levels. This assessment was repeated over 4 days.

3. RESULTS

3.1. Method development for the analysis of total BMAA

3.1.1. Sample preparation

Using the ALG-06 environmental sample, the initial analysis revealed almost 100% suppression of BMAA signals due to a very large excess of amino acids generated from the acid hydrolysis of proteins and peptides in the cyanobacteria sample. Further investigation showed that arginine was co-eluting with BMAA and was likely causing the suppression observed.

Attempts to chromatographically separate BMAA from arginine were unsuccessful and a sample clean-up step was added to the protocol. The strong cation exchange SPE method reduced the concentration of arginine in the BMAA fraction to a level where the BMAA signal was no longer saturated (Figure 3 and Figure 4).

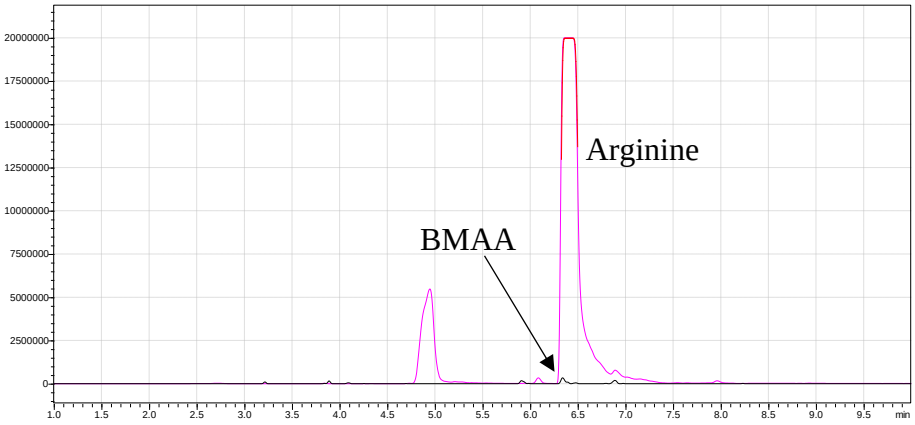

Figure 3. LC-MS/MS chromatograms showing β-N-methylaminoalanine (BMAA) and arginine before solid-phase extraction clean-up.

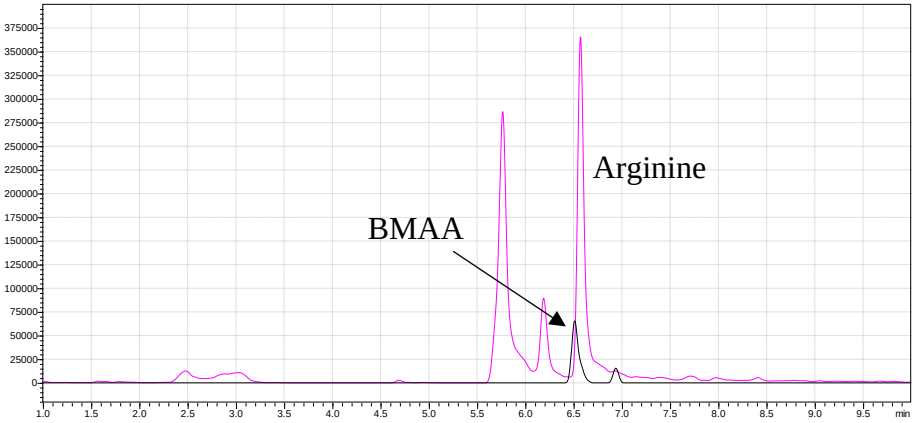

Figure 4. LC-MS/MS chromatograms showing β-N-methylaminoalanine (BMAA) and arginine following solid-phase extraction clean-up.

3.1.2. Mass spectrometric analysis

The switch from analysing free BMAA to total BMAA presented challenges due to the large amounts of amino acids produced during the acid hydrolysis step. This was observed as an increase in baseline interferences and a reduced potential to generate charged BMAA in the source of the mass spectrometer – the result being a decrease in the signal for BMAA.

While method performance partially improved with the SPE clean-up (Section 3.1.1), to further counter these effects the multiple-reaction monitoring (MRM) transitions used to measure total BMAA were reinvestigated in the hydrolysed sample matrix. As described in Cawthron Report 3796 (van Ginkel et al. 2022), the MRM transition *m/z* 119.1>44 was less selective than the MRM transition *m/z* 119.1>76 for distinguishing BMAA from its isomers, especially BAMA. However, to gain the sensitivity required for the total BMAA analysis, it was necessary to change the quantitation channel to *m/z* 119.1>44. Increasing the collision energy from 5 eV to 10 eV provided additional signal, enabling a lower LoD and LoQ than would have been otherwise possible. The *m/z* 119.1>76 transition was then used as a confirmation channel for the total BMAA analysis. The high selectivity of the new confirmation MRM channel provided a robust approach for distinguishing BMAA from interfering compounds.

Table 4. Multiple-reaction monitoring transitions and collision energies for free and total β-N-methylaminoalanine (BMAA).

Setting	Free BMAA	Total BMAA
Quantitation channel	*m/z* 119.1>76	*m/z* 119.1>44
Collision energy (eV)	10	10
Confirmation channel	*m/z* 119.1>44	*m/z* 119.1>76
Collision energy (eV)	5	10

3.2. Analytical validation for the analysis of total BMAA

3.2.1. Selectivity

To obtain maximum sensitivity for BMAA in cyanobacterial hydrolysates while retaining selectivity between BMAA and BAMA, the quantitation and confirmation MRM channels for BMAA were reversed for the analysis of total BMAA (see Section 3.1.2 for details). When the same concentrations of BMAA and BAMA were analysed, the signal from the BMAA was 60-fold stronger than the signal from BAMA (Figure 5). To have a substantial impact on the selectivity, BAMA concentrations would need to be 50 times higher than BMAA in a sample. As noted in Cawthron

Report 3796 (van Ginkel et al. 2022), the other common BMAA isomers (DAB and AEG) were chromatographically separated from BMAA (Figure 5).

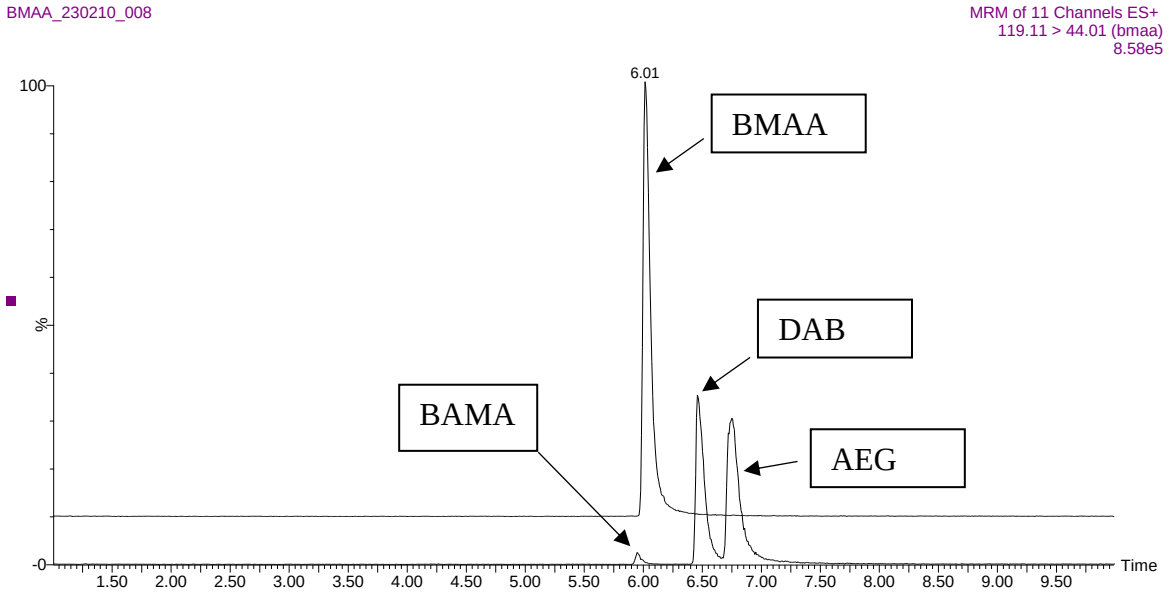

Figure 5. LC-MS/MS chromatograms for the β-N-methylaminoalanine (BMAA) quantitation transition (*m/z* 119.1>44), where BMAA was analysed (top chromatogram) and BMAA isomers analysed (bottom chromatogram) – β-amino-N-methylalanine (BAMA), diaminobutyric acid (DAB) and N-(2-aminoethyl)-glycine (AEG).

An investigation was undertaken to confirm that spiking of an acid hydrolysed cyanobacteria sample (ALG-06) with $^{13}C^{15}N_2$-BMAA would not result in an interfering signal in the BMAA channels. No signal was observed at 6.16 min in the BMAA MRM transitions (*m/z* 119.1>76 and *m/z* 119.1>44; Figure 6); however, it was present in the $^{13}C^{15}N_2$-BMAA MRM transitions (*m/z* 122.1>77 and *m/z* 122.1>46; Figure 6).

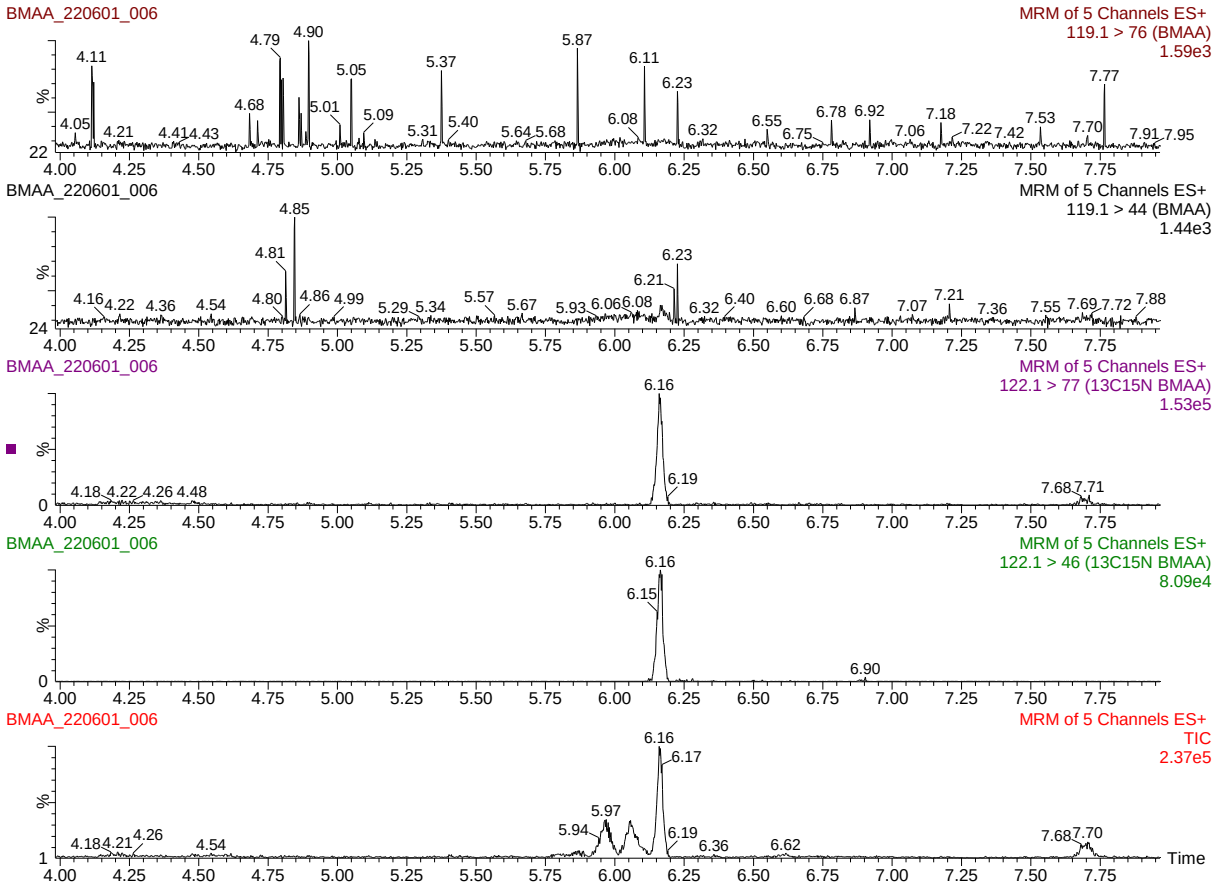

Figure 6. LC-MS/MS chromatograms for a hydrolysate of the ALG-06 sample spiked with 10 ng/mL of $^{13}C_{15}N_2$-β-N-methylaminoalanine (BMAA). From top to bottom: quantitation and confirmation BMAA transitions, two transitions for $^{13}C^{15}N_2$-BMAA, and total ion chromatogram for all transitions.

3.2.2. Linearity

BMAA external standards were acquired over a range of 1–50 ng/mL. The internal standard ($^{13}C^{15}N_2$-BMAA) was incorporated at a fixed level of 10 ng/mL to correct for matrix effects from samples. The working calibration range of 1–50 ng/mL produced a linear response for BMAA, as demonstrated by the coefficient of determination (R^2) being > 0.99. An example of the calibration curve is provided in Figure 7.

The 'calibration curve' (the ratio of BMAA LC-MS/MS response to $^{13}C^{15}N_2$-BMAA LC-MS/MS response) was consistent over the four batches analysed during the validation study (Table 5). While variation was typically observed in the LC-MS/MS response for $^{13}C^{15}N_2$-BMAA (10% RSD), the overall calibration curve for BMAA was highly consistent (2.2% RSD) due to it being relative to the internal standard. The R^2 for the BMAA LC-MS/MS response was high (> 0.99) and consistent (0.1% RSD; Table 5).

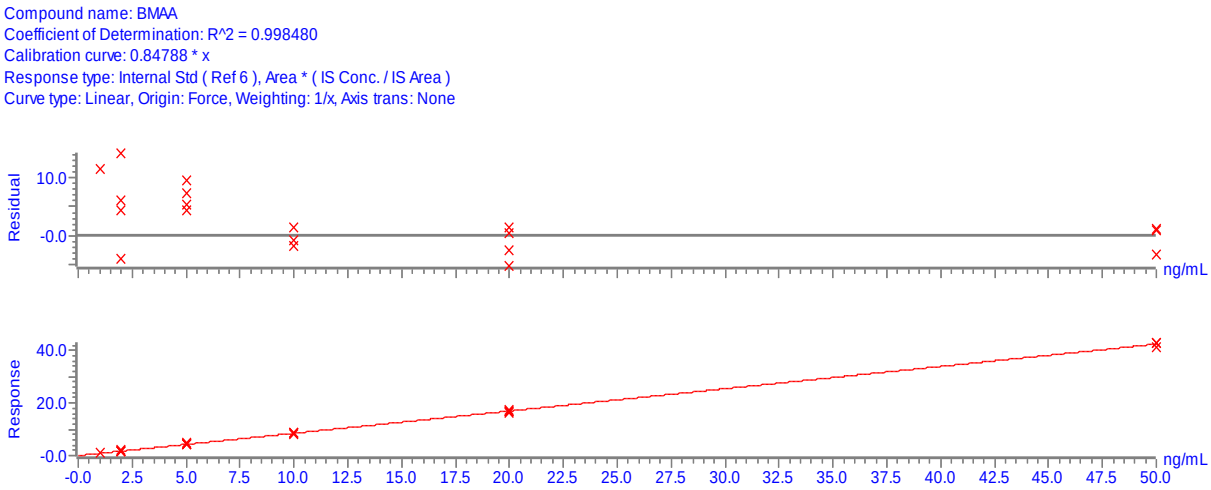

Figure 7. Typical calibration curve for β-N-methylaminoalanine (BMAA) using $^{13}C^{15}N_2$-BMAA as an internal standard, showing linear working range of 1–50 ng/mL.

Table 5. Calibration performance for four validation batches of the total β-N-methylaminoalanine (BMAA) method, including mass spectrometric response for the internal standard ($^{13}C^{15}N_2$-BMAA), calibration R^2 for BMAA and the response of the calibration curve (the ratio of BMAA response to $^{13}C^{15}N_2$-BMAA response).

Validation batch	Response ($^{13}C^{15}N_2$-BMAA)	R^2 (for BMAA)	Calibration curve (BMAA/$^{13}C^{15}N_2$-BMAA)
1	1,671	0.999	0.886
2	1,431	0.999	0.869
3	1,441	0.997	0.889
4	1,317	0.998	0.848
Average	1,465	0.998	0.873
Standard deviation	148	0.001	0.019
Relative standard deviation	10%	0.1%	2.2%

3.2.3. Limit of detection

An estimation of the theoretical LoD and LoQ was undertaken based on the procedure of Magnusson and Örnemark (2014). This procedure describes using the standard deviation of at least eight replicate sample analyses undertaken under repeatability conditions to calculate the sensitivity of an analytical method. Each cyanobacterial matrix evaluated was fortified at a level of 500 µg/kg (equivalent to 5 ng/mL; the mid-calibration level). Sixteen samples were each analysed using the ALG-06 and spirulina samples, and eight samples were each analysed using CAWBG-104 and CAWBG-114 samples.

For each dataset, the standard deviation was calculated. For the datasets containing 16 replicates, the LoD was defined as 2.6 times the standard deviation, and for datasets containing eight replicates, the LoD was defined as 3 times the standard deviation (Magnusson and Örnemark 2014). For all samples, the LoQ was defined as 10 times the standard deviation. The evaluation determined that the theoretical LoD for the total BMAA analysis was 93–247 µg/kg and the theoretical LoQ was 310–950 µg/kg (Table 6).

Table 6. Theoretical limit of detection and limit of quantitation calculated for the two cyanobacteria samples and two cyanobacterial strains (SD = standard deviation; RSD = relative standard deviation; LoD = limit of detection; LoQ = limit of quantitation).

Sample	SD (µg/kg)	RSD	LoD (µg/kg)	LoQ (µg/kg)
ALG-06	46	9.2%	120	460
Spirulina	95	17.6%	247	950
CAWBG-104	55	11.0%	165	550
CAWBG-114	31	6.2%	93	310

While relatively consistent theoretical LoD and LoQ values were observed for the ALG-06, CAWBG-104 and CAWBG-114 samples, the spirulina sample had distinctly higher values than the other three matrices. Based on these results, a limit of reporting (LoR) was set at 300 µg/kg. At this level, positive samples can be distinguished from blank samples. However, quantitation at the lower levels may be inaccurate for some samples.

3.2.4. Precision

Within-day repeatability (short-term precision; RSD_r) was generated for the two cyanobacteria samples. Four replicate samples were fortified at a level of 500 µg/kg of BMAA, and within-day repeatability varied between 5.9% and 10.9% RSD for the ALG-06 sample, and 5.8% and 36.1% RSD for the spirulina sample (Table 7). Through the evaluation of multiple batches of replicate samples (four replicates each), the RSD_r was calculated as per the International Organization for Standardization (2019). The RSD_r for the ALG-06 sample was 8.2% and for the spirulina sample it was 20.7% (Table 7).

Between-day reproducibility (intermediate precision; RSD_R) was generated for the two cyanobacteria samples. Each was fortified with BMAA at 500 µg/kg. Four replicates of each sample were prepared on four different days (n = 16) and RSD_R was calculated as per the International Organization for Standardization (2019). For both samples, the RSD_R was similar to the RSD_r observed for the same sample. Spirulina (19.9%

RSD_R) performed substantially worse than ALG-06 (9.4% RSD_R), although this was primarily due to more variable results in the first batch.

Table 7. Precision data determined through the analysis of four batches of fortified cyanobacteria samples (each batch had four replicates; SD = standard deviation; RSD = relative standard deviation; LoD = limit of detection; LoQ = limit of quantitation).

Sample	Batch	Average	SD	RSD [a]	RSD_r [b]	RSD_R [c]
ALG-06	1	4.72	0.51	10.9%	**8.2%**	**9.4%**
	2	5.23	0.37	7.1%		
	3	5.36	0.32	5.9%		
	4	4.84	0.43	8.8%		
Spirulina	1	4.41	1.59	36.1%	**20.7%**	**19.9%**
	2	4.83	0.64	13.2%		
	3	4.51	0.26	5.8%		
	4	5.32	0.94	17.6%		

[a] Within-batch repeatability determined using four replicates.
[b] Short-term precision (RSD_r) was calculated as per the International Organization for Standardization (2019).
[c] Intermediate precision (RSD_R) was calculated as per the International Organization for Standardization (2019).

3.2.5. Accuracy

At the start of this validation no known positive cyanobacteria reference material was available to evaluate the method accuracy. Therefore, accuracy was determined from fortification experiments on each of the two cyanobacteria samples. Fortification was performed at three levels (200 µg/kg, 500 µg/kg and 1,000 µg/kg). Average recoveries in both the ALG-06 and the spirulina samples were reasonable at all fortification levels (95–125% recovery; Table 8). While this evaluation does not assess hydrolysis efficiency (how much of the BMAA is liberated from sample proteins), it does provide a measure of analyte recovery through the sample preparation procedure.

Table 8. Average percentage recovery for β-N-methylaminoalanine (BMAA) in fortified cyanobacteria samples.

Sample	BMAA fortification level (µg/kg)		
	200 [a]	**500** [b]	**1,000** [c]
ALG-06	125%	101%	101%
Spirulina	107%	95%	106%

[a] n = 4; [b] n = 16; [c] n = 4

3.3. Cultured cyanobacteria results

Of the 32 cyanobacterial strains and two cyanobacterial samples tested, only one contained total BMAA above the standard LoR of 300 µg/kg (*Planktothrix* sp. CAWBG-35, 2,600 µg/kg; Table 9). The other *Planktothrix* sp. sample tested (CAWBG-59) also contained a low level of total BMAA (100 µg/kg). Background noise in the *Planktothrix* sp. CAWBG-59 sample was sufficiently low, allowing clear identification of BMAA at this low level. The detected concentration of 100 µg/kg was also similar to the theoretical LoDs determined for ALG-06 (120 µg/kg) and *Leptolyngbya* sp. CAWBG-114 (93 µg/kg) in the sensitivity experiments (see Table 6), adding confidence to the low-level detection in *Planktothrix* sp. CAWBG-59.

For several strains, the LoR had to be adjusted to 1,000 µg/kg because of matrix interference (*Microcystis aeruginosa* CAWBG-617) or insufficient sample to use the standard sample amount of 25 mg (*Microcystis* sp. CAWBG-706 and *Scytonema* sp. CAWBG-630; Table 9).

Table 9. Total β-N-methylaminoalanine (BMAA) results for cultured cyanobacteria strains and cyanobacteria samples (NA = not applicable; samples in bold are where BMAA was detected above the limit of reporting, LoR).

#	Cyanobacterial taxonomy (sample specifics)	CICCM code	BMAA (µg/kg)
1	Spirulina (commercially grown *Arthrospira platensis*)	NA	< 300
2	ALG-06 (*Microcoleus autumnalis*-dominated sample)	NA	< 300
3	*Calothrix* sp.	CAWBG-78	< 300
4	*Cuspidothrix issatschenkoi*	CAWBG-02	< 300
5	*Cuspidothrix issatschenkoi*	CAWBG-31	< 300
6	*Desmonostoc muscorum*	CAWBG-702	< 300
7	*Dolichospermum lemmermannii*	CAWBG-680	< 300
8	*Geitlerinema* sp.	CAWBG-533	< 300
9	*Leptolyngbya* sp.	CAWBG-114	< 300
10	*Limnothrix* sp.	CAWBG-541	< 300
11	Merismopediaceae family	CAWBG-749	< 300
12	*Microcoleus autumnalis*	CAWBG-24	< 300
13	*Microcoleus autumnalis*	CAWBG-635	< 300
14	*Microcystis aeruginosa*	CAWBG-617	< 1,000*
15	*Microcystis* sp.	CAWBG-11	< 300
16	*Microcystis* sp.	CAWBG-706	< 1,000**
17	*Microcystis wesenbergii*	CAWBG-647	< 300
18	*Nodularia spumigena*	CAWBG-704	< 300
19	*Nodularia spumigena*	CAWBG-712	< 300
20	*Nodularia spumigena*	CAWBG-714	< 300
21	*Nostoc* sp.	CAWBG-29	< 300
22	*Nostoc* sp.	CAWBG-627	< 300
23	*Oscillatoria* sp.	CAWBG-104	< 300
24	*Phormidium* sp.	CAWBG-28	< 300
25	***Planktothrix* sp.**	**CAWBG-35**	**2,600**
26	*Planktothrix* sp.	CAWBG-59	< 300
27	*Pseudoanabaena* sp.	CAWBG-49	< 300
28	*Scytonema* cf. *crispum*	CAWBG-72	< 300
29	*Scytonema* cf. *crispum*	CAWBG-524	< 300
30	*Scytonema* sp.	CAWBG-630	< 1,000**
31	*Scytonema* sp.	CAWBG-632	< 300
32	Synechococcaceae family	CAWBG-734	< 300
33	Synechococcaceae family	CAWBG-747	< 300
34	*Wilmottia murrayi*	CAWBG-95	< 300

* The LoR was increased due to interferences in the matrix, causing increased uncertainty quantifying BMAA.
** The LoR was increased due to insufficient sample availability.

4. DISCUSSION

4.1. Method development and validation

A method was successfully developed and validated for the determination of total BMAA in cyanobacterial strains and samples. As anticipated, the measurement of total BMAA was more difficult than that for free BMAA (see van Ginkel et al. 2022). This was primarily due to the formation of large amounts of free amino acids from the hydrolysis of peptides and proteins in the cyanobacteria. This reduced the mass spectrometric ionisation potential for BMAA and interfered with the detection and quantitation of BMAA. The presence of arginine proved to be the most significant interference as it co-eluted with BMAA and was present in quantities many millions of times higher than BMAA. A cation exchange SPE clean-up method was developed to remove most of the arginine by leveraging the differences in isoelectric points of the two compounds. This SPE method also separated neutral or acidic compounds away from the BMAA, reducing background noise and potential interferences.

To obtain an LoD low enough to provide meaningful results, the MRM transition used for quantitation of total BMAA was re-optimised for sensitivity. By changing to the *m/z* 119.1>44 MRM transition and increasing the collision energy, a 10-fold increase in signal without any significant increase in background noise was achieved. Although some specificity was sacrificed between the detection of BMAA and BAMA (an isomer of BMAA), the method was still highly selective due to the 50-fold better sensitivity for BMAA over BAMA and the inclusion of the *m/z* 119.1>76 confirmation channel.

Based on an evaluation of the theoretical LoDs and LoQs for four validation samples, an LoR of 300 µg/kg was established. Because this was lower that the theoretical LoQs determined for some samples, the accuracy of the reported value may be diminished at low levels in some samples. However, at these levels it will still be possible to distinguish positive results from blanks and report these with a high degree of certainty. The accuracy for both validation samples was generally good, and recoveries ranged from 95% to 125%. The 125% recovery observed in the ALG-06 sample was in the lowest fortification level (200 µg/kg), reinforcing comments above about inaccuracy at levels around the limit of reporting (300 µg/kg). At the higher fortification levels (500 µg/kg and 1,000 µg/kg), accuracy was very good (95–106% recovery).

The precision of the method varied between the two validation matrices used. The spirulina sample performed significantly worse than the ALG-06 sample, providing RSD_r and RSD_R of 20.7% and 19.9%, respectively. Poor analytical performance for total BMAA using a commercial spirulina sample has been reported previously (Tymm et al. 2021); therefore, the use of laboratory-cultured cyanobacteria for validation experiments might be beneficial for any future work. Laboratory-cultured

cyanobacteria were not used in the present study due to the time delays involved in producing sufficient cyanobacterial material for the validation study.

In summary, the analytical method described here is fit for use as a research tool to measure total BMAA in cyanobacteria at concentrations ≥ 500 µg/kg. Below 500 µg/kg, it provides a useful detection tool down to concentrations of approximately 100 µg/kg, although measured results at this level may not be accurate.

4.2. Total BMAA analysis of cyanobacteria strains and samples from Aotearoa New Zealand

To evaluate the prevalence of BMAA in cyanobacteria from Aotearoa New Zealand, 32 cyanobacteria strains and two other cyanobacteria samples (environmental benthic mat material dominated by *Microcoleus autumnalis* and commercially grown spirulina – *Arthrospira platensis*) were analysed using the new method. Only two *Planktothrix* sp. strains were positive. Higher levels of total BMAA were detected in *Planktothrix* sp. CAWBG-35 (2,600 µg/kg) compared to *Planktothrix* sp. CAWBG-59, where only trace levels were detected (100 µg/kg). Because these two *Planktothrix* strains were isolated from the same environmental sample, the vast differences in BMAA concentrations suggest inter-strain differences in BMAA production. Similar observations have been previously documented for cyanotoxins such as anatoxins (Wood et al. 2012) and microcystins (Puddick et al. 2019).

Based on previous work where BMAA was not detected in cyanobacteria when HILIC-MS/MS direct analysis methods were adopted (e.g., McCarron et al. 2014; Réveillon et al. 2014; Beach et al. 2015; Réveillon et al. 2015; Faassen et al. 2016), the low detection rate of BMAA (6% were positive) in cyanobacteria from Aotearoa New Zealand was not surprising. The total BMAA levels detected in *Planktothrix* sp. CAWBG-35 (2,600 µg/kg) were slightly higher than those previously detected in strains of marine microalgae using direct analysis by HILIC-MS/MS (320–750 µg/kg; Réveillon et al. 2015). Comparisons with studies using derivatisation methodologies have not been provided here because of the likelihood for false positives and overestimations of BMAA concentrations when using these methods (see Cawthron Report 3796 for more information; van Ginkel et al. 2022).

The detection of BMAA in only *Planktothrix* sp. suggests that the investigation of other cyanobacterial taxa might be required to properly understand the prevalence of BMAA in cyanobacteria from Aotearoa New Zealand. The freshwater cyanobacterial taxa evaluated here covered 20 genera and ≥ 23 species, with a range of planktonic and benthic cyanobacteria observed in lakes and rivers around Aotearoa New Zealand. The samples included bloom-forming taxa (e.g. *Dolichospermum*, *Microcoleus*, *Microcystis*, *Nodularia*) and widespread taxa such as picocyanobacteria (Merismopediaceae and Synechococcaceae). However, there are many other

freshwater cyanobacteria genera found in Aotearoa New Zealand that were not analysed during the current study. While some additional cyanobacterial taxa are available in the CICCM, the collection does not include every cyanobacterial species observed in fresh waters around Aotearoa New Zealand.

In Europe, *Planktothrix* forms blooms in many lakes, but in Aotearoa New Zealand it is not a particularly prevalent cyanobacterial taxon in lakes. Benthic *Planktothrix* sp. has been observed in some rivers in Aotearoa New Zealand (although it is not frequently reported), where it has been associated with at least one dog death due to microcystin production (at the Waitaki River in South Canterbury; Wood et al. 2010). In these river environments it grows as thin, bright green films on the bottom of the pools that can form to the side of rivers and in backwater regions of rivers.

While BMAA observed in cycad seeds has been linked to *Nostoc* sp. growing symbiotically with the cycad plant (Cox et al. 2003), during the current study BMAA was not detected in the *Nostoc* sp. CAWBG-29 or CAWBG-627, or the closely related *Desmonostoc muscorum* CAWBG-702. Cox and Sacks (2002) detected 300 µg/kg (expressed as 0.3 µg/g in the article) of free BMAA in *Nostoc* sp. cultures isolated from the coralloid roots of cycad plants. Since total BMAA is expected in higher concentrations than free BMAA, the absence of total BMAA at concentrations > 300 µg/kg in three *Nostoc* cultures suggests that BMAA production is not widespread throughout all *Nostoc* spp. However, Cox and Sacks (2002) did use analytical methodology that adopted AQC derivatisation for their study, rather than direct analysis, so the BMAA results reported there might be higher than the true value. In agreement with other studies, BMAA was not detected in *Arthrospira* spp. (spirulina; Jiang et al. 2013; McCarron et al. 2014) during the current study.

4.3. Other considerations relating to BMAA

Despite the low detection rates of BMAA in freshwater cyanobacteria from Aotearoa New Zealand, previous work using HILIC-MS/MS direct analysis methods have detected BMAA in marine mussels, oysters, phytoplankton, periphyton and diatom cultures from overseas (Réveillon et al. 2014; Beach et al. 2015; Réveillon et al. 2015; Faassen et al. 2016; Réveillon et al. 2016). The detection of BMAA in shellfish, phytoplankton and diatom cultures suggests that algae (and potentially cyanobacteria) are the source of BMAA observed in shellfish. Investigations of marine microalgae cultures might reveal other BMAA-producing organisms.

While outside the scope of the current study, toxicology studies definitively demonstrating the negative health effects of BMAA are still lacking. Current health concerns related to BMAA are primarily based on the high rates of ALS-PDC observed in the Chamorro people of Guam (Cox and Sacks 2002; Cox et al. 2003) rather than direct toxicological evidence. Therefore, no World Health Organization

guideline values have been developed for BMAA to date. A summary of toxicology evidence and assumptions relating to BMAA can be found in Chorus and Welker (2021).

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7. APPENDICES

Appendix 1. Cyanobacteria strains from the Cawthron Institute Culture Collection of Microalgae (CICCM), including the growth parameters and harvesting methods used

Cyanobacterial taxonomy	CICCM code	Description	Media	Growth method	Harvest method
Calothrix sp.	CAWBG-78	Filamentous, benthic.	MLA	5	1
Cuspidothrix issatschenkoi	CAWBG-02	Filamentous, planktonic.	MLA	3	1
Cuspidothrix issatschenkoi	CAWBG-31	Filamentous, planktonic.	MLA	3	1
Desmonostoc muscorum	CAWBG-702	Filamentous, benthic.	MLA	2	1
Dolichospermum lemmermannii	CAWBG-680	Filamentous, colonial, planktonic.	MLA	1	1
Geitlerinema sp.	CAWBG-533	Filamentous, benthic.	MLA	1	1
Leptolyngbya sp.	CAWBG-114	Filamentous, benthic.	F/2	2	1
Limnothrix sp.	CAWBG-541	Filamentous, benthic.	MLA	2	1
Merismopediaceae family	CAWBG-749	Pico, coccoid, planktonic.	MLA	1	1
Microcoleus autumnalis	CAWBG-24	Filamentous, benthic.	MLA	1	3
Microcoleus autumnalis	CAWBG-635	Filamentous, benthic.	MLA	2	1
Microcystis aeruginosa	CAWBG-617	Coccoid, single-cell, planktonic.	MLA	3	1
Microcystis sp.	CAWBG-11	Coccoid, colonial, planktonic.	MLA	4	4
Microcystis sp.	CAWBG-706	Coccoid, colonial, planktonic.	MLA	1	1
Microcystis wesenbergii	CAWBG-647	Coccoid, colonial, planktonic.	MLA	5	1
Nodularia spumigena	CAWBG-704	Filamentous, colonial, planktonic.	MLA	3	1

Cyanobacterial taxonomy	CICCM code	Description	Media	Growth method	Harvest method
Nodularia spumigena	CAWBG-704	Filamentous, colonial, planktonic.	MLA	3	4
Nodularia spumigena	CAWBG-704	Filamentous, colonial, planktonic.	MLA	4	3
Nostoc sp.	CAWBG-29	Filamentous, benthic.	MLA	1	1
Nostoc sp.	CAWBG-627	Filamentous, benthic.	MLA	2	2
Oscillatoria sp.	CAWBG-104	Filamentous, benthic.	F/2	5	1
Phormidium sp.	CAWBG-28	Filamentous, benthic.	MLA	2	1
Planktothrix sp.	CAWBG-35	Filamentous, bi-phasic.	MLA	2	1
Planktothrix sp.	CAWBG-59	Filamentous, bi-phasic.	MLA	2	1
Pseudoanabaena sp.	CAWBG-49	Filamentous, benthic.	MLA	2	1
Scytonema cf. *crispum*	CAWBG-72	Coarse filament, benthic.	MLA	2	2
Scytonema cf. *crispum*	CAWBG-524	Coarse filament, benthic.	MLA	1	1
Scytonema sp.	CAWBG-630	Coarse filament, benthic.	MLA	1	3
Scytonema sp.	CAWBG-632	Coarse filament, benthic.	MLA	1	1
Synechococcaceae family	CAWBG-734	Pico, coccoid, planktonic.	MLA	2	1
Synechococcaceae family	CAWBG-747	Pico, coccoid, planktonic.	MLA	1	1
Wilmottia murrayi	CAWBG-95	Filamentous, benthic.	MLA	1	1

Notes: See Bolch and Blackburn (1996) for MLA medium and Guillard and Ryther (1962) for F/2 medium. Growth parameters are described in Table 1. Harvesting methods are described in Section 2.1.1.

Appendix 2. Acquisition parameters for the analysis of total β-N-methylaminoalanine by hydrophilic interaction chromatography-tandem mass spectrometry

Hydrophilic interaction chromatography-tandem mass spectrometry instrument parameters

Parameter	**Description / composition**
Column	Waters Acquity UPLC BEH Amide (1.7 µm, 2.1 × 150 mm)
Mobile phases	A – MQ water 0.2% formic acid B – ACN 0.2% formic acid
Flow rate	0.4 mL/min
Injection volume	2 µL
Column oven	30 °C
Mass Lynx LC parameter set	BMAA_Grad
Mass Lynx MS Tune file	BMAA.ipr
Ionisation mode	+ESI
Cone gas flow	150 L/h
Desolvation gas flow	800 L/hr
Desolvation temperature	400 °C
Source temperature	150 °C
Capillary	1.5 kV
Cone	20 V
Mass Lynx MS Experiment file	BMAA_internal_std_.MRM
Mode	MRM
MassLynx Quan method	TargetLynx (BMAA_MRM.mdb)
Quantitation mode	Area
Calibration regression	Linear, force origin, Weighting 1/X

Chromatographic mobile phase gradient

Time (min)	% A	% B	Flow rate (mL/min)	Gradient
0	10	90	0.4	Initial
10	50	50	0.4	6
15	10	90	0.4	1

Multiple-reaction monitoring (MRM) acquisition parameters

Compound	Acquisition time (min)	Parent ion (*m/z*)	Daughter ion (*m/z*)	Collision energy (eV)	Dwell time (s)
BMAA	1–10	119.1	44.0	10	0.02
	1–10	119.1	76.0*	10	0.02
Isomers	1–10	119.1	102.0	10	0.02
$^{13}C^{15}N_2$-BMAA	1–10	122.1	77.0	10	0.02

* MRM qualifier channel.