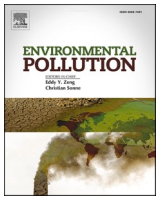
Environmental Pollution 363 (2024) 125081

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol



Bioaccumulation of β-methylamino-L-alanine (BMAA) by mussels exposed to the cyanobacteria *Microcystis aeruginosa*☆

Siobhan J. Peters [a], Simon M. Mitrovic [b], Kenneth J. Rodgers [b], David P. Bishop [a,*]

[a] Hyphenated Mass Spectrometry Laboratory, Faculty of Science, The University of Technology Sydney, Ultimo, NSW, 2007, Australia
[b] School of Life Sciences, Faculty of Science, The University of Technology Sydney, Ultimo, NSW, 2007, Australia

ARTICLE INFO

Keywords:
Cyanotoxins
Bioaccumulation
BMAA
Bioindicators
Freshwater mussels

ABSTRACT

Cyanobacterial blooms are increasingly common in aquatic environments, raising concerns about the health impacts associated with the toxins they produce. One of these toxins is β-methylamino-L-alanine (BMAA), a neurotoxin linked to neurodegenerative diseases. Monitoring BMAA levels in the environment is challenging due to trace concentrations and complex matrices, and new approaches are needed for assessing exposure risk. In this laboratory study, Australian freshwater mussels, *Velesunio ambiguus*, were exposed to a BMAA-producing cyanobacterium, *Microcystis aeruginosa*, to assess its accumulation of the toxin over time. A sample preparation and analysis method was developed to allow accurate quantification of BMAA in the mussels at concentrations as low as 0.4 ng/g. Mussels exposed to *M. aeruginosa* accumulated BMAA, with concentrations increasing over the exposure period. Rapid depuration occurred after exposure to the cyanobacterium ended, with concentrations of BMAA quickly returning to pre-exposure levels. These results demonstrate the potential for mussels to be used as bioindicators in the field for monitoring BMAA levels over time, where rapid depuration is unlikely.

1. Introduction

Cyanobacteria are ubiquitous photosynthetic microorganisms that, whilst present in some of the most inhabitable extremes on Earth, are most commonly found in aquatic environments (Díez et al., 2014). Rapid proliferation events can occur when water conditions are favourable for cyanobacteria growth, leading to large-scale cyanobacterial blooms. These conditions have occurred more often in recent years, mainly due to anthropogenic influences such as eutrophication and climate change (O'Neil et al., 2012). Cyanobacterial blooms can be harmful for many reasons, impacting both physical and chemical water quality. One of the main concerns are the health risks associated with the toxins that cyanobacteria can produce (Huisman et al., 2018). These toxins, collectively known as cyanotoxins, include hepatotoxic cyclic peptides (e.g. microcystins), neurotoxic amino acids (e.g. β-methylamino-L-alanine (BMAA)) and alkaloids (e.g. anatoxins and saxitoxins), and endotoxic lipopolysaccharides.

β-Methylamino-L-alanine (BMAA) is a non-protein amino acid thought to be primarily produced by cyanobacteria, as well as some species of diatoms and dinoflagellates (Cox et al., 2005; Jiang et al., 2014a; Jiang et al., 2014b). BMAA is linked to high incidences of global neurodegenerative disease (Caller et al., 2018). Although the acute neurotoxic effects of BMAA have been extensively observed both in vivo and in vitro (Karamyan et al., 2008; Chan et al., 2012; Main et al., 2016; Potjewyd et al., 2017), the exact mechanism contributing to neurodegeneration remains unclear. Chronic exposure to BMAA is believed to contribute to neurodegeneration (Caller et al., 2018), but validating this hypothesis through experimental modelling has proven challenging. BMAA is frequently found alongside its structural isomers 2,4-diaminobutyric acid (2,4-DAB) and N-(2-aminoethyl)glycine (AEG), both of which display their own neurotoxicity (Schneider et al., 2020; Main et al., 2018a; Metcalf et al., 2015). AEG's toxicity remains contentious, with conflicting reports in the literature (Schneider et al., 2020; Main et al., 2018a). Populations residing near water bodies prone to cyanobacterial blooms face heightened risks of BMAA exposure. Exposure can be through direct contact with contaminated water, inhalation of aerosols, or consuming contaminated food (Cox et al., 2005; Jonasson et al., 2010; Li et al., 2024; Lance et al., 2018; Scott et al., 2018; Banack et al., 2015; Metcalf et al., 2023). Exposure through food sources is exacerbated by BMAA's ability to bioaccumulate, creating higher

☆ This paper has been recommended for acceptance by Dr. Sarah Harmon.

* Corresponding author.
E-mail address: david.bishop@uts.edu.au (D.P. Bishop).

https://doi.org/10.1016/j.envpol.2024.125081
Received 12 July 2024; Received in revised form 6 September 2024; Accepted 4 October 2024
Available online 5 October 2024

concentrations in food sources such as seafood (Lance et al., 2018; Brand et al., 2010; Regueiro et al., 2017; Dunlop et al., 2019).

Determining levels of BMAA exposure can be challenging. This results from a combination of difficulties with analytical methods, low concentrations, and complex sample matrices (Bishop et al., 2020). Many of the studies analysing BMAA in the environment focus on cyanobacterial samples (Bishop et al., 2020; Esterhuizen-Londt et al., 2011a; Yan et al., 2017). This approach is problematic, as cyanobacterial levels may not reflect levels of human exposure to BMAA. Detection of BMAA in environmental cyanobacteria samples is also varied, with some studies failing to detect BMAA despite analysing large sample sizes (Monteiro et al., 2017; Wang et al., 2023), while others report its presence in multiple samples (Violi et al., 2019; Faassen et al., 2009; Esterhuizen-Londt et al., 2011b; Błaszczyk et al., 2021). This inconsistency between detections is common even within a genus or species and has led to difficulties in developing an understanding of the role BMAA plays in the environment, as well as the conditions that are required for its production. Analysis of cyanobacteria samples also requires a high biomass for detection and is only representative of the time point at which the sample was collected. Cyanobacterial cell numbers can change rapidly due to movement by wind or currents (Wang et al., 2016; Wu et al., 2013; Chen et al., 2023), and concentrations of BMAA in cyanobacteria can quickly change across a bloom event (Peters et al., 2022). Both of these could lead to over- or under-estimation of BMAA exposure and risk when a single sampling period is used, influencing the reliability of limited spatial sampling designs.

Studies of BMAA concentrations in environmental waters have had limited success. Pre-concentration is needed to increase the levels of BMAA in water to detectable amounts, but most studies still have insufficient sensitivity to detect the toxin. Two papers have successfully detected BMAA in natural, unspiked water, with a majority of detections being less than 2 ng/L (Vo et al., 2019; Cao et al., 2019). In other studies, the reported limits of detection for BMAA in water range between 10 and 5000 ng/L (Yan et al., 2017; Roy-et al., 2015; Faassen et al., 2013; Abbes et al., 2022), meaning these other methods would not have been sensitive enough to be able to detect BMAA in the majority of positive water samples (Yan et al., 2017; Vo et al., 2019; Cao et al., 2019; Roy-et al., 2015; Faassen et al., 2013; Abbes et al., 2022). Robust and sensitive water monitoring for BMAA is important as water presents a probable route of toxicity. However, water analysis is still limited to providing information at a single time point that was sampled.

Bioindicators offer a solution to analysing trace concentrations of toxins or pollutants in water as they can bioaccumulate the target analytes into higher concentrations, and may offer insights into concentration levels over extended periods of time. Bivalves, such as mussels, have been used extensively in marine and freshwater environments as bioindicators of environmental pollution, including heavy metals, persistent organic pollutants, and microplastics (Li et al., 2021; Qu et al., 2018; Viarengo et al., 1991; Azizi et al., 2018; Carro et al., 2014; Farrington et al., 1983). They make particularly effective bioindicators due to their filter-feeding behaviour, sedentary lifestyle, long lifespan, wide distribution and bioaccumulation potential (Viarengo et al., 1991). Some BMAA analyses have focussed on aquatic animals which have accumulated BMAA, often within the context of assessing the risk of exposure through food consumption (Lance et al., 2018; Regueiro et al., 2017; Sandhu et al., 2024; Jiang et al., 2014c). While these studies provide valuable insights, they typically offer limited temporal information and lack controlled exposure durations. BMAA has been identified in filter-feeding bivalves such as mussels and oysters, which consume phytoplankton including cyanobacteria (Lance et al., 2018; Jiang et al., 2014c; Réveillon et al., 2014). BMAA concentrations in mussels has been a focus of some recent studies due to concerns regarding direct human consumption (Regueiro et al., 2017), bioaccumulation potential into livestock through use in feed (Kim and Rydberg, 2020), and as potentially useful bioindicators for BMAA levels in aquatic environments (Lepoutre et al., 2020a; Lepoutre et al., 2020b; Downing et al., 2014; Baptista et al., 2015). Utilising mussels as bioindicators for BMAA analysis presents an alternative to the complications of both water and phytoplankton analysis, whilst providing information over larger periods of time, potentially enabling more effective monitoring of chronic exposure.

Laboratory experiments have demonstrated the uptake of dissolved BMAA in mussels (Lepoutre et al., 2020a; Lepoutre et al., 2020b; Downing et al., 2014), but have required a relatively high concentration of ≥5 μg/L, which is much higher than levels detected in natural water samples. One study also observed some BMAA uptake in marine mussels fed cyanobacteria, but had limited data points and failed to show a clear trend (Baptista et al., 2015). These studies provide some pretext for using mussels as bioindicators of BMAA in the environment. The use of mussels to analyse microcystins (MCs) and other cyanotoxins in laboratory settings and in the environment suggests this approach may be useful (Amorim et al., 1999; Pires et al., 2004; Camacho-Muñoz et al., 2021; Vasconcelos, 1995; Saker et al., 2004).

Unfortunately, the most common mussels used in bioaccumulation studies, including those used for cyanotoxins, are unsuitable for use in Australia. One of the more commonly used species, *D. polymorpha*, is an invasive species in many parts of the world, and Australia has a particularly sensitive environment. Therefore, selecting a native species, such as *Velesunio ambiguus*, is essential for establishing mussels as environmental bioindicators in Australia. *V. ambiguus* has a widespread distribution across much of eastern Australia and is characterised by its hardiness and limited mobility, suggesting its potential suitability for bioaccumulation studies. Here we aimed to analyse the uptake and elimination of BMAA in *V. ambiguus* mussels exposed to toxin-producing cyanobacterium, *Microcystis aeruginosa,* and to develop new sample preparation methods to increase sensitivity and enable BMAA detection in the complex sample matrix. This was done to assess the viability of mussels as a bioindicator of cyanotoxin levels over time.

2. Methods & materials

2.1. Reagents and materials

L-BMAA hydrochloride (BMAA HCl, ≥97%) and microcystin RR-YR-LR mix (5 μg/mL in methanol) standards for LC-MS/MS analysis were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). L-2,4-diaminobutyric acid dihydrochloride (2,4-DAB 2HCl, ≥95%) and N-(2-aminoethyl)-glycine (AEG, ≥97%) standards were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). The internal standards were BMAA D3 (d3BMAA, ≥98%) from Eurofins Technology (Dandenong South, Vic, Australia), D-2,4-diaminobutyric-2,3,3,4,4-d5 acid dihydrochloride (d5DAB 2HCl, ≥66%) from CDN Isotopes (Pointe-Claire, QC, Canada), and nodularin from Sigma-Aldrich (Castle Hill, NSW, Australia). The d5DAB internal standard had multiple deuteration states, and d3 was found to be the most abundant. Methanol and acetonitrile were both LiChrosolv® hypergrade for LC-MS, and purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Analytical reagent grade sodium hydroxide, hydrochloric acid, trichlorocaetic acid, 1-propanol, propyl-chloroformate, 3-methylpyridine, chloroform and 2,2,4-trimethylpentane (isooctane) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). HPLC grade ethyl acetate and ammonium hydroxide were also purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Solid phase extraction cartridges were Oasis MCX, 3 cc 60 mg, from Waters (Rydalmere, NSW, Australia)

2.2. Mussel acclimation

Individual *V. ambiguus* were purchased from Australian Aquatic Biological (Karuah, NSW, Australia) and acclimated in the laboratory until experimental procedures began. Upon arrival, mussels were placed in a 100 L tank and covered in “copper-free” water overnight. The following day mussels were distributed into aerated tanks with 1 L

water/mussel. During this acclimation period, mussels were fed 3.5 × 10^6 chlorella/mussel/day. Chlorella was supplemented with 40% algae hikari (ground to 100 μm) when algal suspension was unavailable at 2 mg/mussel/day. Water temperatures were kept at 20 ± 1 °C, a pH of 7.5 ± 0.1, and dissolved oxygen at 8.0 ± 0.5 mg/L.

2.3. Exposure and depuration

A total of 70 mussels (63 ± 5 mm) were used for the exposure experiment. At the beginning of the experiment, the mussels were transferred to 5 separate tanks, each holding 14 mussels and 14L of copper-free water. During the exposure period, mussels were fed 2 × 10^7 cells/mussel/day of *Microcystis aeruginosa MASH01-AO5* at a frequency of twice a week (Fig. 1). This cyanobacterium was chosen for the exposure experiment as it had previously been found to produce BMAA (Violi et al., 2019; Main et al., 2018b; Downing et al., 2011), and it was quantified with the same methods as described for the mussel tissue. The free fraction of the cyanobacterium had a concentration of 0.65 ng BMAA/g, while the insoluble protein fraction had no detectable BMAA. 15 mussels were transferred to a control tank that was fed chlorella as described in the acclimation throughout the experiment. After the exposure period of 56 days, mussels were left to depurate for 56 days. At the beginning of depuration, mussels were moved to fresh, clean tanks. For the duration of the experiment, half water changes were completed at every sampling period, or once every 2 weeks, whichever was sooner, with water volume adjusted to maintain 1 L water/mussel.

2.4. Sampling

Samples were collected on days 0, 3, 7, 14, 21, 28, 42 and 56 during the exposure period, and on days 59, 63, 70, 77, 88, 112 during the depuration period (Fig. 1). At each of the sampling points, one mussel was randomly selected from each of the five exposure tanks. Sacrificed samples were shucked, weighed and sliced before being freezing at −80 °C. Samples were freeze-dried, weighed to obtain dry weights and homogenised by mortar and pestle. Samples were then stored at −80 °C until prepared for analysis.

2.5. Cell lysis and protein precipitation

Approximately 50 mg of dry mussel tissue was weighed into a 2 mL Eppendorf tube, along with 1 mL of 10% trichloroacetic acid (TCA) and 20 μL of an internal standard (IS) mix containing 500 ng/mL of d_5DAB and d_3BMAA. Samples underwent probe sonication (Qsonica Newtown, CT, USA) twice for 1.5 min at 50% intensity and were kept on ice in between. The samples were left at 4 °C for 4 h to enable protein precipitation before being centrifuged at 3000×*g* for 20 min. The resulting supernatant was transferred into a 2 mL Eppendorf tube. The remaining pellet was transferred to a glass shell vial by two additional 200 μL washes of 10% TCA. Both additional washes were combined with the original supernatant and labelled as the "soluble fraction". The soluble fraction was freeze dried overnight at −80 °C, 0.1 mbar before being reconstituted in 400 μL 20 mM HCl and transferred to 0.2 μm membrane spin filter tubes (Ultrafree-MC LG Centrifugal 0.2 μL PTFE Membrane Filter).

2.6. Protein hydrolysis

Shell vials containing the protein pellets were transferred to hydrolysis vials containing 1 mL 6M HCl. The sealed hydrolysis vials were purged under vacuum and replaced with nitrogen 3 times. They were left in an oven at 110 °C for 16 h to undergo hydrolysis by HCl vapour. After 16 h, the vials were briefly left to cool, and the pressure was released. The hydrolysed pellets were then transferred to 0.2 μm membrane spin filter tubes (Ultrafree-MC LG Centrifugal 0.2 μL PTFE Membrane Filter) by reconstituting with 380 μL 20 mM HCL and 20 μL IS mix. The spin filter tubes from both this insoluble protein fraction as well as the soluble fraction were then centrifuged at 8000×*g* for 1 h. Extracts were either stored at −20 °C or proceeded directly to the next stage.

2.7. Extraction

Sample clean-up was completed using Waters MCX SPE cartridges (3 cc, 60 mg). Cartridges were conditioned with 1 mL 10% NH_4OH in MeOH followed by 2 mL MeOH. Equilibration was by 1 mL 10 mM HCL and 200 μL of sample was loaded with an additional 800 μL 10 mM HCl. The loaded cartridges were washed first with 1 mL 10 mM HCl, followed by 2 mL MeOH. Cartridges loaded with the protein fraction samples underwent an additional 1.5 mL wash with 2% NH_4OH to remove the additional interferences found in this matrix, but this step was not needed for the free fraction. All cartridges were dried under vacuum before elution in 2 mL 10% NH_4OH. The samples were dried under a gentle nitrogen stream before being reconstituted in 100 μL of 20 mM HCl, ready for derivatisation.

2.8. Derivatisation

All samples and standards were derivatised using propyl-

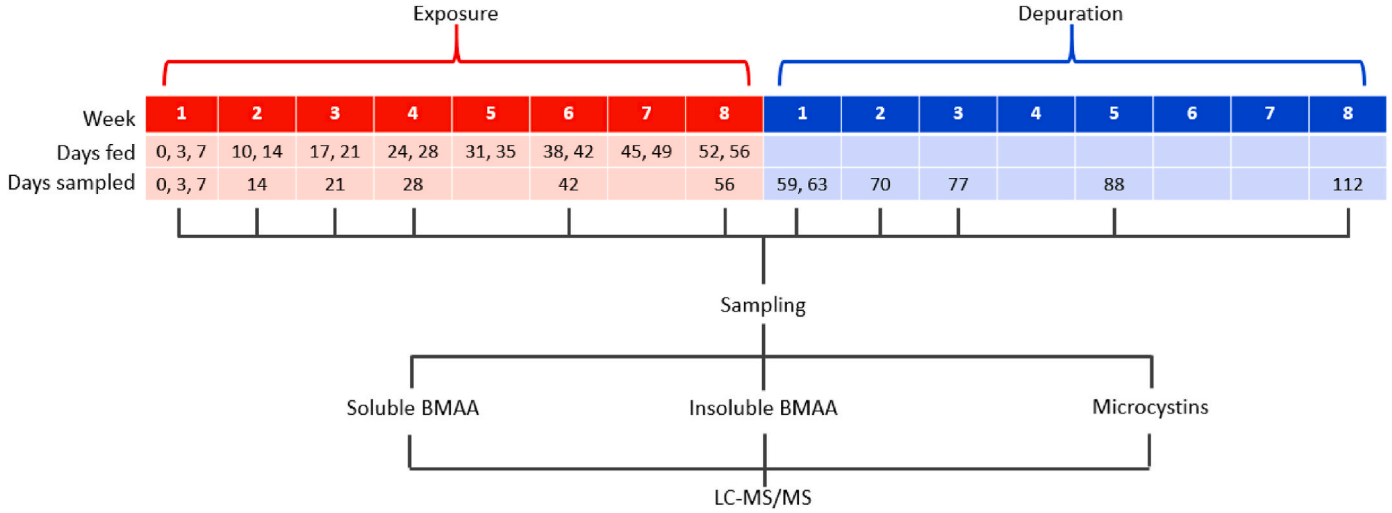


Fig. 1. Feeding and sampling timeline and experimental flow chart for the exposure and depuration periods.

chloroformate. This was done by the addition of 100 μL of 20% 3-methylpyridine in 1-propanol, followed by the addition of 50 μL of propyl-chloroformate/chloroform/isooctane (18%/72%/10%). The mixture was vortexed thoroughly to ensure full derivatisation. 100 μL of ethyl acetate was then added, and the solution was vortexed again and left for a minute for the layers to separate. The top layer was transferred to an LC vial insert and an additional 100 μL of ethyl acetate was added to the sample for a second wash. After vortexing, the top layer was combined with the first extract. The combined extracts were dried down under a gentle stream of nitrogen and were finally reconstituted 100 μL of the starting mobile phase solvent, which consisted of 50% MeOH in water.

2.9. Analysis by LC-MS/MS

Standards were made ranging from 0.025 to 1000 ng/mL of both BMAA and DAB, and with a final concentration of 25 ng/mL of d_3BMAA and d_5DAB. All dilutions used 20 mM HCl. Standards were derivatised prior to LC-MS/MS using the same protocol as the samples.

The LC-MS/MS method was optimised on a Shimadzu Nexera UC UHPLC system coupled to a Shimadzu LCMS-8060 triple quadrupole mass spectrometer (Rydalmere, NSW, Australia). Chromatographic separation was achieved using an Agilent Zorbax Eclipse Plus RRHD column (2.1 × 50 mm, 1.8 μm) equipped with a 5 mm guard column containing the same stationary phase. The column temperature was maintained at 40 °C and flow rate was set to 0.8 mL/min. The starting mobile phase consisted of 50% ultrapure water with 0.1% formic acid (A) and 50% methanol with 0.1% formic acid (B). The starting mobile phase composition was maintained for the first 5 min, after which time all target analytes had eluted and the mobile phase was increased to 90% B until 7 min to wash the column. The mobile phase then returned to the initial conditions to equilibrate before the next injection for 1.5 min. Each sample underwent duplicate 10 μL injections.

The MS/MS was run in positive mode, with optimised parameters including an interface voltage of 3 kV and interface temperature set at 300 °C. The desolvation temperature was maintained at 526 °C, with the desolvation line temperature at 250 °C. Nebulising gas flow was set to 2.90 L/min, with heating gas at a flow rate of 10.00 L/min. A heat block temperature of 400 °C was employed, along with a drying gas flow rate of 10.00 L/min. Multiple reaction monitoring (MRM) mode was used with specific ion transitions and collision energies detailed in Table 1. Data analysis was performed using Shimadzu's Lab Solutions. The sample preparation and instrument methods were validated by assessing linearity, instrument and sample detection limits (LODs), accuracy, and precision.

2.10. Microcystin extraction and analysis

For the analysis of MCs in mussel tissue, an additional 50 mg of each sample was weighed out into 2 mL Eppendorf tubes. 990 μL of 75% MeOH was added to each sample, along with 10 μL of 1 μg/mL of nodularin as an internal standard. The samples were briefly vortexed and left in a sonicator bath for 30 min. Samples then underwent probe sonication at 50% intensity for 1 min, before being filtered (Ultrafree-MC LG Centrifugal 0.2 μL PTFE Membrane Filter) and transferred to vials for LC-MS/MS analysis. Microcystin standards included MC-RR, MC-YR and MC-LR, and were made in a range of 0.1 ng/mL and 500 ng/mL, and had a final concentration of 10 ng/mL of nodularin. All dilutions were done in 75% MeOH in water.

Analysis took place using modified method from Pravadali-Cekic et al. (2023) with the same LC-MS/MS system described for the BMAA analysis. Separation was completed using an Agilent Zorbax Eclipse Plus RRHD column (2.1 × 100 mm, 1.8 μm), with a column temperature of 18 °C and a flow rate of 0.65 mL/min. The starting mobile phase was 70% A (ultrapure water + 0.1% formic acid) and 30% B (acetonitrile + 0.1% formic acid), increasing to 32.5% B over 4 min. The mobile phase was held for 0.5 min at 32.5% B before increasing to 90% B for 1.5 min. The system then returned to starting conditions to equilibrate for 2 min before the next injection. 5 μL was injected of each sample in duplicate.

2.11. Statistical analysis

Statistical analyses were completed using Microsoft excel and Python. Mussels with undetectable analyte levels were considered to have a concentration of 0 for statistical analyses. Grubbs tests were used to exclude any outliers. Student's t-tests were used to assess difference in data sets.

3. Results & discussion

A highly sensitive and accurate sample preparation method for the analysis of BMAA was developed (Table 2), which enabled the detection of BMAA in mussel tissue at concentrations as low as 0.4 ng/g. This is over 2500x more sensitive than recent methods employed for similar sample matrices (Lepoutre et al., 2020a; Lepoutre et al., 2020b) which have limits of detection in the μg/g range. This is also the first method to use non-commercial propyl-chloroformate derivatisation. The limits of detection in samples vary, with the soluble fraction being 20 times lower than the hydrolysed TCA insoluble fraction. An additional wash during SPE was required for the insoluble protein fraction to help remove some of the interferences present in this fraction. However, detection limits were still impacted by the more complex sample matrix. This can be seen in the chromatograms in Fig. 2, where the intensity of the peak corresponding to BMAA has a lower intensity in the spiked sample matrix than in the standard mix. This decrease is not replicated in the 2,4-DAB peak, as all soluble fractions of the mussel samples had high levels of 2,4-DAB present and we were therefore unable to utilise a blank matrix for 2,4-DAB. Instead, this peak corresponds to the combined response from the naturally occurring and spiked 2,4-DAB in the sample. The impact of

Table 1
MRM parameters for 2,4-DAB and BMAA.

	Precursor Ion (m/z)	Product ion (m/z)	Collision energy (eV)
2,4-DAB	333.2	231.1	−15
		187.1	−19
		99.1$^+$	−11
D_5 2,4-DAB[a]	336.2	276.2	−10
		190.2	−20
		234.2$^+$	−15
BMAA	333.2	187.1	−20
		159.2	−18
		73.2$^+$	−14
D_3 BMAA[a]	336.2	190.1	−20
		162.1	−18
		76.1$^+$	−14

[a] Denotes internal standards. + denotes the ion transition used for quantification.

Table 2
Method validation results. % recovery and % accuracy are calculated from 5 ng/mL standards that underwent SPE. Recovery is from comparing peak areas of the MRM used for quantification. Accuracy is normalised using the respective internal standard. Repeatability is the peak area %RSD of 7 repeat injections of a 10 μg/L standard across a single run.

	BMAA	DAB
RETENTION TIME (MIN)	4.08 ± 0.04	2.75 ± 0.02
LINEARITY	>0.999	>0.99
LINEAR RANGE (NG/ML)	0.025–500	0.1–500
METHOD LOD (NG/ML)	0.025	0.1
SAMPLE LOD SOLUBLE (NG/ML)	0.05	0.5
SAMPLE LOD INSOLUBLE (NG/ML)	1	1
% RECOVERY	22%	38%
% ACCURACY	105 ± 5%	103 ± 2%
REPEATABILITY	2.3%	3.8%

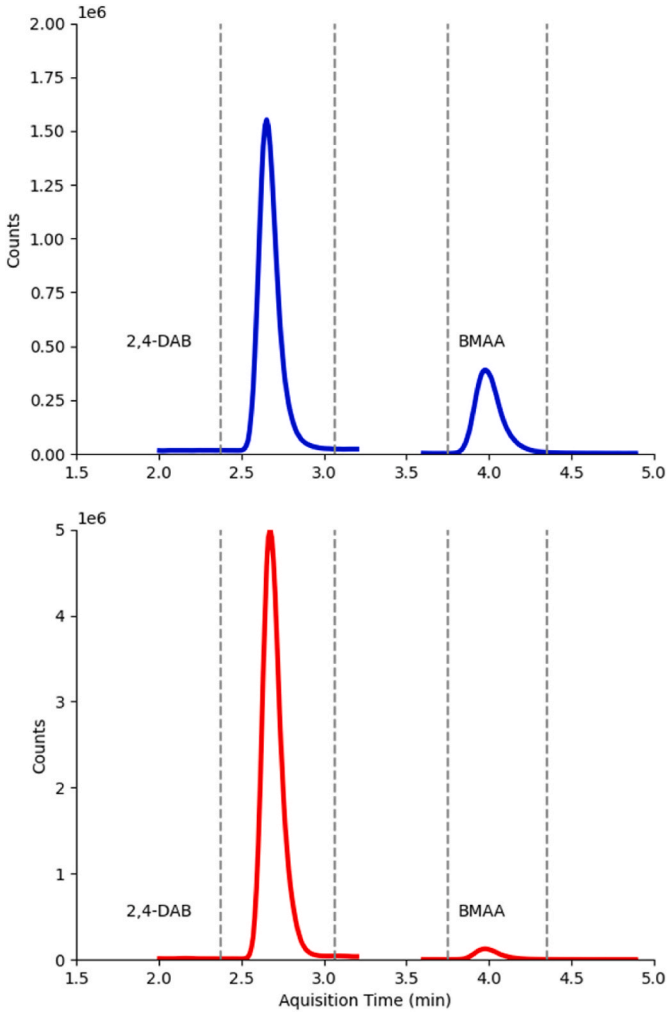


Fig. 2. Representative chromatograms of 2,4-DAB and BMAA using the respective quantification MRM for each analyte. The blue line (above) is a chromatogram of a standard containing 5 ng/mL of 2,4-DAB and BMAA. The red line (below) is of a representative soluble fraction of a mussel sample spiked with 5 ng/mL of 2,4-DAB and BMAA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the matrix is further exemplified by spiked recoveries where only peak areas were compared, showing percentage recoveries from 22 to 38% (Table 2). A high level of accuracy was achieved by normalising to the appropriate internal standard regardless of any loss during sample preparation or suppression from the sample matrix. The difference between the two fractions also contributed to the choice to keep the fractions separate, rather than analysing total BMAA, as the small amounts that were detected in the free fraction would not have been detectable when in the full sample matrix.

Mussel samples from across all time points had detectable levels of BMAA in the soluble fraction, with concentrations ranging between 1.36 ng/mussel and 7.34 ng/mussel, including on day 0 which was prior to exposure. There was a significant difference between the start and end of the exposure period (*t*-test, $p < 0.05$) and a positive linear correlation between BMAA concentration and length of exposure was observed ($r^2 = 0.77$, Fig. 3). Following the exposure period, BMAA concentrations rapidly returned to pre-exposure levels but complete elimination did not occur, with mussels sampled on the final day of depuration still having detectable levels of BMAA. This suggests that the full elimination of BMAA from the mussels would require a much longer period, or that BMAA does not get fully removed from the system. The incomplete

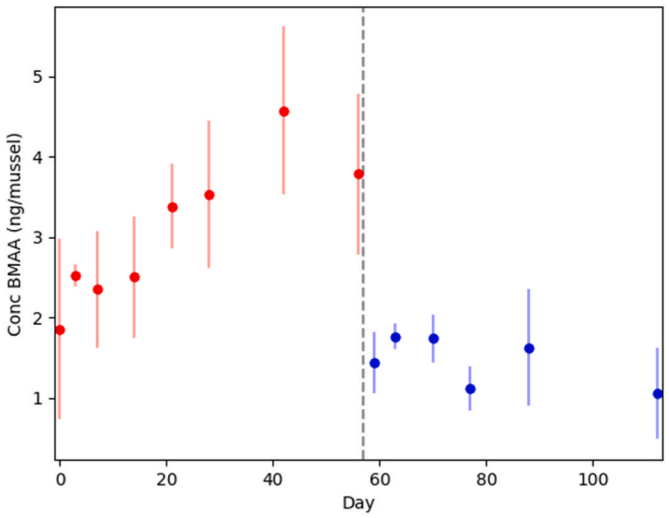


Fig. 3. Concentration of BMAA in mussels over the duration of the experiment. Dots denote the mean concentration and error bars are standard error. Red is the exposure period and blue is the depuration period, with the grey dashed line showing the end of the exposure period. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

removal of BMAA could explain the presence of BMAA in the controls, as there is no significant difference between the day 0 control samples and any of the samples from the depuration period. This suggests exposure could have occurred prior to obtaining the mussels. This is consistent with some other mussel accumulation studies which assessed uptake of free BMAA dissolved in the water, where they also found detectable levels of BMAA in the control mussels (Lepoutre et al., 2020a; Lepoutre et al., 2020b). However, these studies did not observe the rapid and non-linear drop in BMAA concentration that was seen in our study, with one showing a half-life of between 10 and 20 days for mussels exposed to dissolved BMAA, depending on the mussel species and concentration of BMAA used for exposure (Lepoutre et al., 2020b). Whilst the rapid depuration of BMAA could be a limitation of in situ use, the immediate removal of all sources of BMAA from the system would not be replicated in environmental settings.

The current study did not detect BMAA in the insoluble protein fraction. However, it is possible that BMAA accumulated in this fraction, but at levels below the limit of detection. Previous studies investigating mussel uptake of dissolved BMAA in water detected BMAA in this fraction, but at notably lower concentrations than the soluble free fraction (Lepoutre et al., 2020a; Lepoutre et al., 2020b). These studies also used concentrations of BMAA higher than have been detected in water, resulting in overall higher concentrations than observed in our findings. The similar uptake patterns observed here and those using dissolved BMAA suggest that mussels may uptake cyanobacterial BMAA in a manner similar to freely available BMAA in the water column. This could be beneficial for using mussels as bioindicators of total BMAA exposure, as it is likely for both forms to be present in environmental conditions. However, it contradicts the suggestion that the primary mode of BMAA uptake was through direct diffusion rather than through the digestive tract (Lepoutre et al., 2020a).

The maximum concentration of BMAA in *V. ambiguus* occurred on day 42 of exposure, the second-to-last sampling point in the exposure period. Despite the expectation that the highest concentration would occur at the final sampling point due to the longest exposure, statistical analysis (*t*-test, $p < 0.05$) showed no significant difference between the two points. Any observable variance can, therefore, be attributed to biological variability rather than the extent of exposure. As the cyanobacterium used for this exposure experiment, *M. aeruginosa*, also produces MCs, this was used to further validate the observed trend of BMAA

concentrations (Supplementary Fig. 1). A similar pattern was observed for the MC-LR concentration, with the highest concentration being present in the second last sampling point of the exposure period (Fig. 4). As this change is consistent amongst the different cyanotoxins, it is likely that the mussels sampled on this date were more active than those taken on the 56th day. An alternative explanation has been proposed in earlier works looking at accumulation and depuration dynamics of MC-LR in zebra mussels, which similarly saw a drop in MC-LR concentration towards the end of an exposure period (Pires et al., 2004). This was suggested to be a result of mechanisms that enabled the mussels to reduce exposure to cyanotoxins (Pires et al., 2004). Whilst the decrease in concentration occurred earlier than seen in this study, the concentrations of MC-LR were much higher, peaking at 16.3 μg/g, which could have caused an earlier response to reduce further exposure. Therefore, the observed decrease in both BMAA and MC-LR between the 42nd and 56th day may be due to reduced feeding, potentially to prevent the accumulation of high MC-LR concentrations. The species of mussel used in this study, V. ambiguus, are floodplain mussels that are able to remain inactive or dormant for significant periods of time (Rogers et al., 2010). Dormancy and adaptability to different conditions could affect the consistency of mussel filtration rates and lead to variation in exposure between individual mussels. This could be an impact factor for the interindividual differences observed across each time point, and similar variation levels have been seen in previous V. ambiguus metal bioaccumulation studies (Jones et al., 1979; Kibria et al., 2023).

The quick but incomplete depuration of BMAA was also mirrored by MC-LR concentrations, with MC-LR dropping by over 70% within 3 days of depuration. This is similar to what has been seen in previous studies looking at MC accumulation in mussels (Pires et al., 2004; Camacho--Muñoz et al., 2021; Vasconcelos, 1995; Chen et al., 2007) but the present work is the first study to look at MC levels in *V. ambiguus*. One notable difference observed between the two classes of cyanotoxin is that MC-LR was not detectable in *V. ambiguus* prior to exposure, whilst BMAA was present in 3 out of 5 of the mussels at the start of the study. This could be from limited prior exposure, or a result of more efficient removal mechanisms.

The predictable pattern of uptake of both BMAA and MC-LR by *V. ambiguus* makes this species a suitable bioindicator for both cyanotoxins, making it a potentially useful tool for monitoring in Australian freshwater environments. Using these mussels in regular monitoring practices could have substantial benefits, providing additional temporal information over traditional monitoring practices. This could include being used as a warning system, showing information from a bloom event that could have otherwise been missed or looking at exposure risk over longer periods of time. The results also suggest that other mussel species globally could serve as effective bioindicators for cyanotoxins, potentially enhancing monitoring practices in various freshwater and marine environments worldwide. This could facilitate the incorporation of cyanotoxin monitoring into already established programs as well as utilising bioindicators in new monitoring strategies.

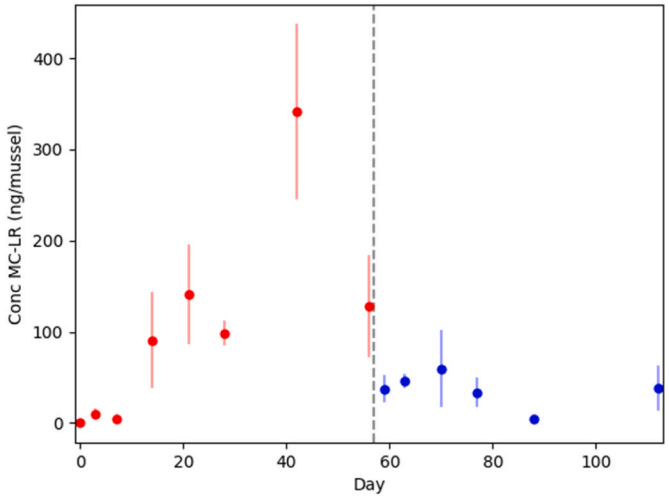


Fig. 4. Concentration of MC-LR in mussels over the duration of the experiment. Dots denote the mean concentration and error bars are standard error. Red is the exposure period and blue is the depuration period, with the grey dashed line showing the end of the exposure period. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The presence of 2,4-DAB in both soluble and insoluble fractions, with concentrations much higher than BMAA (Supplementary Table 1), presents a limitation for the use of *V. ambiguus* as a bioindicator of total cyanotoxin levels. The lack of correlation between 2,4-DAB concentration and exposure suggests that its origin is not solely from cyanobacteria, especially as the concentrations were much higher than the *M. aeruginosa* that was used for the exposure experiment. Unlike BMAA, 2,4-DAB is not limited to being produced by phytoplankton, and instead can be produced by bacteria, resulting in 2,4-DAB being more widespread. The incompatibility of *V. ambiguus* for 2,4-DAB monitoring shows the importance of assessing suitability for individual cyanotoxins, and places further emphasis on the need for highly selective instrumental methods to ensure confident differentiation between the two isomers. AEG was also monitored prevent misidentification of BMAA (Supplementary Fig. 2). However, neither of the internal standards provided acceptable accuracy for this isomer so AEG was not quantified.

The rapid depuration of both BMAA and MC-LR observed in this study also present a limitation for using *V. ambiguus* as a bioindicator of cyanotoxins. The quick decline in toxin levels could lead to an underestimation of cyanotoxin exposure if sampling occurs after the peak of the bloom has passed. However, the experimental conditions of complete starvation during the depuration period do not replicate environmental conditions. Cyanobacterial blooms typically decline gradually, and residual cyanobacteria, along with toxins released into the water during lysis, may persist after the bloom has peaked (Romo et al., 2013). Additionally, studies on toxins produced by marine phytoplankton and taken up by mussels showed a phase-lag, with peak toxin concentrations occurring in mussels approximately two months after the peak in phytoplankton, further indicating a delay in toxin clearance (Kim et al., 2023). The more gradual decline that would be seen in the environment may mitigate the rapid decline in toxin levels seen in this controlled laboratory experiment. The findings of this study still suggest that well-timed and frequent sampling is necessary during bloom events to ensure toxin levels are assessed accurately. While *V. ambiguus* demonstrates its suitability as a bioindicator for short-term monitoring of cyanotoxins, limitations could arise when attempting to monitor toxin levels over longer periods, where there would be more variability of cyanobacteria biomass. The rapid depuration period also allows for accurate identification of the end of a risk event, provided sampling is conducted regularly.

Future works should expand to assessing other cyanotoxins to assess the viability of *V. ambiguus* or other mussels as bioindicators for more comprehensive cyanotoxin monitoring, and look at deployment into environmental waterways prone to cyanobacterial blooms to assess optimal sampling periods and to better understand the dynamics of cyanotoxin uptake and depuration in environmental settings. Additionally, further studies are needed to investigate the uptake and depuration dynamics with cyanobacteria containing higher concentrations of BMAA, as well as BMAA present in the insoluble protein fraction. Either of these could result in detectable concentrations of BMAA being present in the insoluble hydrolysed fraction of the mussels, which would enable assessing the suitability of this fraction for biomonitoring purposes.

4. Conclusions

This study demonstrates that *V. Ambiguus* mussels have the potential to be used as bioindicators of BMAA concentrations in aquatic environments. BMAA levels increased in the mussels fed BMAA-containing cyanobacterium over the course of the exposure period and returned to pre-exposure levels during the depuration period. A similar trend was seen for MC-LR, making these mussels potentially useful bioindicators of multiple cyanotoxins. A highly sensitive method for detecting BMAA in low concentrations in mussel tissue was also developed. The rapid depuration of BMAA must be considered if these mussels are used in monitoring programs, as sampling plans must be considered so as not to miss a risk event. Future work should consider uptake and depuration dynamics in environmental settings and explore a broader range of cyanotoxins.

CRediT authorship contribution statement

Siobhan J. Peters: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Simon M. Mitrovic:** Writing – review & editing, Funding acquisition, Conceptualization. **Kenneth J. Rodgers:** Writing – review & editing, Funding acquisition, Conceptualization. **David P. Bishop:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

Acknowledgements

DPB, SM, and KR were funded by the Ian Potter Foundation, and SP by an Australian Government Research Training Program Scholarship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.125081.

References

Abbes, S., et al., 2022. Occurrence of BMAA isomers in bloom-impacted lakes and reservoirs of Brazil, Canada, France, Mexico, and the United Kingdom. Toxins 14 (4), 251.
Amorim, Á., Vasconcelos, V.t., 1999. Dynamics of microcystins in the mussel Mytilus galloprovincialis. Toxicon 37 (7), 1041–1052.
Azizi, G., et al., 2018. The use of Mytilus spp. mussels as bioindicators of heavy metal pollution in the coastal environment. A review. J. Mater. Environ. Sci. 9 (4), 1170–1181.
Banack, S.A., et al., 2015. Detection of cyanotoxins, β-N-methylamino-L-alanine and microcystins, from a lake surrounded by cases of amyotrophic lateral sclerosis. Toxins 7 (2), 322–336.
Baptista, M.S., et al., 2015. Assessment of the non-protein amino acid BMAA in Mediterranean mussel Mytilus galloprovincialis after feeding with estuarine cyanobacteria. Environ. Sci. Pollut. Control Ser. 22, 12501–12510.
Bishop, S.L., Murch, S.J., 2020. A systematic review of analytical methods for the detection and quantification of β-N-methylamino-L-alanine (BMAA). Analyst 145 (1), 13–28.
Błaszczyk, A., et al., 2021. Presence of ß-N-methylamino-L-alanine in cyanobacteria and aquatic organisms from waters of Northern Poland; BMAA toxicity studies. Toxicon 194, 90–97.
Brand, L.E., et al., 2010. Cyanobacterial blooms and the occurrence of the neurotoxin, beta-N-methylamino-l-alanine (BMAA), in South Florida aquatic food webs. Harmful Algae 9 (6), 620–635.
Caller, T., Henegan, P., Stommel, E., 2018. The potential role of BMAA in neurodegeneration. Neurotox. Res. 33, 222–226.
Camacho-Muñoz, D., et al., 2021. Rapid uptake and slow depuration: health risks following cyanotoxin accumulation in mussels? Environmental Pollution 271, 116400.
Cao, Y., et al., 2019. Decomposition of β-N-methylamino-L-alanine (BMAA) and 2, 4-diaminobutyric acid (DAB) during chlorination and consequent disinfection byproducts formation. Water Res. 159, 365–374.
Carro, N., et al., 2014. Distribution and trend of organochlorine pesticides in Galicia coast using mussels as bioindicator organisms. Possible relationship to biological parameters. Chemosphere 102, 37–47.
Chan, S.W., et al., 2012. L-DOPA is incorporated into brain proteins of patients treated for Parkinson's disease, inducing toxicity in human neuroblastoma cells in vitro. Exp. Neurol. 238 (1), 29–37.
Chen, J., Xie, P., 2007. Microcystin accumulation in freshwater bivalves from Lake Taihu, China, and the potential risk to human consumption. Environ. Toxicol. Chem.: Int. J. 26 (5), 1066–1073.
Chen, H., et al., 2023. Rapid horizontal accumulation and bloom formation of the cyanobacterium Microcystis under wind stress. Hydrobiologia 850 (1), 123–135.
Cox, P.A., et al., 2005. Diverse taxa of cyanobacteria produce β-N-methylamino-L-alanine, a neurotoxic amino acid. Proc. Natl. Acad. Sci. USA 102 (14), 5074–5078.
Díez, B., Ininbergs, K., 2014. Ecological importance of cyanobacteria. Cyanobacteria: an economic perspective 41–63.
Downing, S., et al., 2011. Nitrogen starvation of cyanobacteria results in the production of beta-N-methylamino-L-alanine. Toxicon 58 (2), 187–194.
Downing, S., et al., 2014. The fate of the cyanobacterial toxin β-N-methylamino-L-alanine in freshwater mussels. Ecotoxicol. Environ. Saf. 101, 51–58.
Dunlop, R.A., Guillemin, G.J., 2019. The cyanotoxin and non-protein amino acid β-methylamino-L-alanine (L-BMAA) in the food chain: incorporation into proteins and its impact on human health. Neurotox. Res. 36 (3), 602–611.
Esterhuizen-Londt, M., Downing, T., 2011a. Solid phase extraction of β-N-methylamino-L-alanine (BMAA) from South African water supplies. WaterSA 37 (4), 523–527.
Esterhuizen-Londt, M., Downing, S., Downing, T., 2011b. Improved sensitivity using liquid chromatography mass spectrometry (LC-MS) for detection of propyl chloroformate derivatised β-N-methylamino-L-alanine (BMAA) in cyanobacteria. WaterSA 37 (2).
Faassen, E.J., et al., 2009. Determination of the neurotoxins BMAA (β-N-methylamino-L-alanine) and DAB (α-, γ-diaminobutyric acid) by LC-MSMS in Dutch urban waters with cyanobacterial blooms. Amyotroph Lateral Scler. 10 (Suppl. 2), 79–84.
Faassen, E.J., Beekman, W., Lürling, M., 2013. Evaluation of a commercial enzyme linked immunosorbent assay (ELISA) for the determination of the neurotoxin BMAA in surface waters. PLoS One 8 (6), e65260.
Farrington, J.W., et al., 1983. US" Mussel Watch" 1976-1978: an overview of the trace-metal, DDE, PCB, hydrocarbon and artificial radionuclide data. Environmental science & technology 17 (8), 490–496.
Huisman, J., et al., 2018. Cyanobacterial blooms. Nat. Rev. Microbiol. 16 (8), 471–483.
Jiang, L., et al., 2014a. Diatoms: a novel source for the neurotoxin BMAA in aquatic environments. PLoS One 9 (1), e84578.
Jiang, L., Ilag, L.L., 2014b. Detection of endogenous BMAA in dinoflagellate (Heterocapsa triquetra) hints at evolutionary conservation and environmental concern. PubRaw Sci 1 (2), 1–8.
Jiang, L., et al., 2014c. Quantification of neurotoxin BMAA (β-N-methylamino-L-alanine) in seafood from Swedish markets. Sci. Rep. 4 (1), 1–7.
Jonasson, S., et al., 2010. Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure. Proc. Natl. Acad. Sci. USA 107 (20), 9252–9257.
Jones, W., Walker, K., 1979. Accumulation of iron, manganese, zinc and cadmium by the Australian freshwater mussel Velesunio ambiguus (Phillipi) and its potential as a biological monitor. Mar. Freshw. Res. 30 (6), 741–751.
Karamyan, V.T., Speth, R.C., 2008. Animal models of BMAA neurotoxicity: a critical review. Life Sci. 82 (5), 233–246.
Kibria, G., et al., 2023. Replacing the use of live mussels with artificial mussels to monitor and assess the risks of heavy metals. Australas. J. Environ. Manag. 30 (1), 127–140.
Kim, S.-Y., Rydberg, S., 2020. Transfer of the neurotoxin β-N-methylamino-l-alanine (BMAA) in the agro–aqua cycle. Mar. Drugs 18 (5), 244.
Kim, M., et al., 2023. Monthly distribution of lipophilic marine biotoxins and associated microalgae in the South Sea Coast of Korea throughout 2021. Sci. Total Environ. 898, 165472.
Lance, E., et al., 2018. Occurrence of β-N-methylamino-l-alanine (BMAA) and isomers in aquatic environments and aquatic food sources for humans. Toxins 10 (2), 83.
Lepoutre, A., et al., 2020a. How the neurotoxin β-N-Methylamino-L-Alanine accumulates in bivalves: distribution of the different accumulation fractions among organs. Toxins 12 (2), 61.
Lepoutre, A., et al., 2020b. Usability of the bivalves Dreissena polymorpha and Anodonta anatina for a biosurvey of the neurotoxin BMAA in freshwater ecosystems. Environmental Pollution 259, 113885.
Li, J., et al., 2021. Where are we? Towards an understanding of the selective accumulation of microplastics in mussels. Environmental Pollution 286, 117543.
Li, M., et al., 2024. How does the neurotoxin β-N-methylamino-L-alanine exist in biological matrices and cause toxicity? Sci. Total Environ., 171255
Main, B.J., Dunlop, R.A., Rodgers, K.J., 2016. The use of L-serine to prevent β-methylamino-L-alanine (BMAA)-induced proteotoxic stress in vitro. Toxicon 109, 7–12.
Main, B.J., Rodgers, K.J., 2018a. Assessing the combined toxicity of BMAA and its isomers 2, 4-DAB and AEG in vitro using human neuroblastoma cells. Neurotox. Res. 33, 33–42.

Main, B.J., et al., 2018b. Detection of the suspected neurotoxin beta-methylamino-l-alanine (BMAA) in cyanobacterial blooms from multiple water bodies in Eastern Australia. Harmful Algae 74, 10–18.
Metcalf, J.S., et al., 2015. Neurotoxic amino acids and their isomers in desert environments. J. Arid Environ. 112, 140–144.
Metcalf, J.S., Banack, S.A., Cox, P.A., 2023. Cyanotoxin analysis of air samples from the great salt lake. Toxins 15 (11), 659.
Monteiro, M., et al., 2017. Screening of BMAA-producing cyanobacteria in cultured isolates and in in situ blooms. J. Appl. Phycol. 29 (2), 879–888.
O'Neil, J.M., et al., 2012. The rise of harmful cyanobacteria blooms: the potential roles of eutrophication and climate change. Harmful algae 14, 313–334.
Peters, S.J., et al., 2022. The changes in cyanobacterial concentration of β-methylamino-L-alanine during a bloom event. Molecules 27 (21), 7382.
Pires, L.D., et al., 2004. Assimilation and depuration of microcystin–LR by the zebra mussel, Dreissena polymorpha. Aquat. Toxicol. 69 (4), 385–396.
Potjewyd, G., et al., 2017. L-β-N-methylamino-l-alanine (BMAA) nitrosation generates a cytotoxic DNA damaging alkylating agent: an unexplored mechanism for neurodegenerative disease. Neurotoxicology 59, 105–109.
Pravadali-Cekic, S., et al., 2023. Simultaneous analysis of cyanotoxins β-N-methylamino-L-alanine (BMAA) and microcystins-rr,-LR, and-YR using liquid chromatography–tandem mass spectrometry (LC-MS/MS). Molecules 28 (18), 6733.
Qu, X., et al., 2018. Assessing the relationship between the abundance and properties of microplastics in water and in mussels. Science of the total environment 621, 679–686.
Regueiro, J., et al., 2017. Dietary exposure and neurotoxicity of the environmental free and bound toxin β-N-methylamino-l-alanine. Food Res. Int. 100, 1–13.
Réveillon, D., et al., 2014. Beta-N-methylamino-L-alanine: LC-MS/MS optimization, screening of cyanobacterial strains and occurrence in shellfish from Thau, a French Mediterranean lagoon. Mar. Drugs 12 (11), 5441–5467.
Rogers, K., Ralph, T.J., 2010. Floodplain Wetland Biota in the Murray-Darling Basin: Water and Habitat Requirements. CSIRO publishing.
Romo, S., et al., 2013. Water residence time and the dynamics of toxic cyanobacteria. Freshw. Biol. 58 (3), 513–522.
Roy-Lachapelle, A., Solliec, M., Sauvé, S., 2015. Determination of BMAA and three alkaloid cyanotoxins in lake water using dansyl chloride derivatization and high-resolution mass spectrometry. Anal. Bioanal. Chem. 407, 5487–5501.
Saker, M.L., et al., 2004. Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel Anodonta cygnea. Toxicon 43 (2), 185–194.
Sandhu, P.K., Solonenka, J.T., Murch, S.J., 2024. Neurotoxic non-protein amino acids in commercially harvested Lobsters (Homarus americanus H. Milne-Edwards). Sci. Rep. 14 (1), 8017.
Schneider, T., et al., 2020. Neurotoxicity of isomers of the environmental toxin L-BMAA. Toxicon 184, 175–179.
Scott, L.L., Downing, S., Downing, T.G., 2018. The evaluation of BMAA inhalation as a potential exposure route using a rat model. Neurotox. Res. 33, 6–14.
Vasconcelos, V.M., 1995. Uptake and depuration of the heptapeptide toxin microcystin-LR in Mytilus galloprovincialis. Aquat. Toxicol. 32 (2–3), 227–237.
Viarengo, A., Canesi, L., 1991. Mussels as biological indicators of pollution. Aquaculture 94 (2–3), 225–243.
Violi, J.P., et al., 2019. Prevalence of beta-methylamino-L-alanine (BMAA) and its isomers in freshwater cyanobacteria isolated from eastern Australia. Ecotoxicol. Environ. Saf. 172, 72–81.
Vo Duy, S., et al., 2019. Analysis of the neurotoxin β-N-methylamino-L-alanine (BMAA) and isomers in surface water by FMOC derivatization liquid chromatography high resolution mass spectrometry. PLoS One 14 (8), e0220698.
Wang, H., et al., 2016. Separation of wind's influence on harmful cyanobacterial blooms. Water Res. 98, 280–292.
Wang, Z.-Q., et al., 2023. Investigation on cyanobacterial production of the proposed neurotoxin β-N-methylamino-L-alanine (BMAA). Water Biology and Security 2 (4), 100208.
Wu, T., et al., 2013. Dynamics of cyanobacterial bloom formation during short-term hydrodynamic fluctuation in a large shallow, eutrophic, and wind-exposed Lake Taihu, China. Environmental science and pollution research 20, 8546–8556.
Yan, B., et al., 2017. Optimization of the determination method for dissolved cyanobacterial toxin BMAA in natural water. Analytical chemistry 89 (20), 10991–10998.