UTS UNIVERSITY OF TECHNOLOGY SYDNEY

# Assessing the viability of the mussel *V. ambiguus* as a bioindicator of the environmental neurotoxin BMAA

**by Siobhan Peters**

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under the supervision of:
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# Certificate of Original Authorship:

I, Siobhan Peters, declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution.

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# List of publications:

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# Abstract

Cyanobacterial blooms are becoming increasingly prevalent in both marine and freshwater environments, raising concerns about the impacts on public health and the environment. One of the main concerns surrounding cyanobacteria is their ability to produce toxins, such as β-methylamino-L-alanine (BMAA), a neurotoxin that has been linked to high incidences of neurodegenerative disease. Levels of BMAA present in the environment are not regulated, and monitoring of BMAA is not currently undertaken by any government authorities. This is a result of uncertainties around its toxicity mechanisms and the complexity of its analysis. This thesis aims to address the problems in current BMAA monitoring approaches and assess the viability of using Australian native freshwater mussels (*Velesunio ambiguus)* as temporal bioindicators of BMAA levels.

BMAA concentrations across a bloom event were assessed to establish temporal trends, as most previous studies sample a single time point. Concentrations were observed to significantly change across relatively short periods, decreasing as the bloom progressed. This highlights the potential for traditional grab sampling to inaccurately estimate the concentrations of BMAA across a bloom, potentially mis-reporting risk.

Mussels are filter feeding bivalves with historical use as long-term bioindicator for pollutants. To utilise mussels as bioindicators of BMAA levels, a highly sensitive method for detecting BMAA in this matrix was required. Different extraction, derivatisation, and analytical methods were compared to optimise the analysis of mussels and enable detections of BMAA as low as 0.4 ng/g. The optimised method utilised solid-phase extraction, derivatisation by propyl-chloroformate, and LC-MS/MS for analysis. The method was 20-500 times more sensitive than those previously reported for mussel analysis.

To assess the viability of *V. ambiguus* as temporal bioindicators of BMAA the uptake and depuration dynamics were assessed via controlled exposure to toxin-producing cyanobacteria. BMAA concentration in mussel tissue

correlated with the length of exposure, and, upon depuration, BMAA quickly returned to pre-exposure levels. This strong trend demonstrates the potential for using mussels as bioindicators of BMAA levels in the environment. To assess this suitability *in situ*, mussels were deployed to two lakes in rural New South Wales, Australia that are prone to regular cyanobacterial blooms.

In one of the two lakes, unexpected weather patterns resulting in colder and wetter summers prevented the formation of the large-scale cyanobacterial blooms that have been previously reported. However, small blooms still occurred and BMAA was detected in the mussels during these cyanobacterial blooms. The other lake had consistently large cyanobacterial blooms, and higher concentrations of BMAA were found in mussels sampled during times of higher cyanobacterial biomass, further demonstrating the suitability of these mussels to be used as tools for BMAA monitoring.

This thesis establishes the need for temporal monitoring of BMAA and the use of freshwater mussels as bioindicators for this use. Further experiments are needed to confirm the uptake patterns of BMAA by *V. ambiguus* in large-scale bloom conditions and could extend the scope to include other pollutants to increase the value of mussels as pollutant monitoring tools.

# Table of Contents

# List of Figures

# List of Tables



# Abbreviations

| | |
|---|---|
| 2,4-DAB | 2,4-diaminobutyric acid |
| ACN | acetonitrile |

| | |
|---|---|
| AEG | N-(2-aminoethyl) glycine |
| ALS-PDC | amyotrophic lateral sclerosis and parkinsonism-dementia complex |
| AQC | 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate |
| BAMA | β-amino-N-methyl-alanine |
| BMAA | β-methylamino-l-alanine |
| cyanoHAB | cyanobacterial harmful algal bloom |
| DNS | dansyl chloride |
| dSPE | dispersive solid phase extraction |
| DW | dry weight |
| ECF | ethyl chloroformate |
| ELISA | enzyme-linked immunosorbent assay |
| ESI | electrospray ionisation |
| FMOC-Cl | 9-fluorenylmethyl chloroformate |
| GC-MS | gas chromatography- mass spectrometry |
| GC-MS/MS | gas chromatography- tandem mass spectrometry |
| GCxGC-TOFMS | two-dimensional gas chromatography- time of flight mass spectrometry |
| GVW | Goulburn Valley Water |
| HAB | harmful algal bloom |
| HCl | hydrochloric acid |
| HILIC | hydrophilic interaction liquid chromatography |
| HPLC | high-performance liquid chromatography |
| HRMS | high resolution mass spectrometry |
| IS | internal standard |

| | |
|---|---|
| LC | liquid chromatography |
| LC-FLD | liquid chromatography- fluorescence detector |
| LC-MS | liquid chromatography-mass spectrometry |
| LC-MS/MS | liquid chromatography-tandem mass spectrometry |
| LOD | limits of detection |
| *m/z* | mass to charge ratio |
| MC | microcystin |
| MC-LR | microcystin-LR |
| MeOH | methanol |
| MRM | multiple reaction monitoring |
| NPAA | non-protein amino acid |
| PCF | propyl-chloroformate |
| RPLC | reverse phase liquid chromatography |
| S/N | signal to noise ratio |
| SPE | solid phase extraction |
| TMS | trimethylsilation |
| WHO | World Health Organization |
| WW | wet weight |

# Chapter 1: Introduction

## 1.1 Cyanobacteria & harmful algal blooms

Cyanobacteria are a large and diverse phylum of photosynthetic, prokaryotic microorganisms with a wide global distribution, inhabiting both aquatic and terrestrial ecosystems.[1] Aquatic cyanobacteria are often referred to as "blue-green algae" as the phycocyanin pigment that most cyanobacteria produce can cause a bluish colour. However, this name is not entirely accurate as cyanobacteria, being prokaryotic organisms, are not true algae. Cyanobacteria are well-adapted to various extreme environments, with some aquatic species able to photosynthesise under low light conditions and tolerate highly saline waters,[2] while terrestrial forms can withstand high levels of ultraviolet radiation and water stress.[3, 4] Cyanobacteria contribute to primary production and nutrient cycling by producing oxygen, fixing atmospheric nitrogen and also serving as a food source for higher trophic levels.[5, 6]

While cyanobacteria are natural and important parts of many ecosystems, certain conditions such as eutrophication,[7] higher temperatures,[8] and decreased river flow rates,[9] can lead to increased growth rates and result in blooms. These ideal conditions for cyanobacterial proliferation are becoming more common, largely as a result of human activities.[10] Agricultural runoff, industrial waste and sewage can increase nutrient loading of nitrogen and phosphorus in waterways, contributing to eutrophication,[11] whilst changing climate conditions and water management impact water temperatures and flow rates.[12] Consequently, cyanobacterial blooms have increased in prevalence and intensity in recent decades.[13]

Cyanobacterial blooms can negatively affect the physical and chemical quality of the water they inhabit, leading to the commonly used terms harmful algal blooms (HABs) or cyanobacterial harmful algal blooms (cyanoHABs). CyanoHABs can reduce the physical and chemical water quality by lowering dissolved oxygen levels (hypoxia and anoxia), which can lead to fish deaths and

increasing turbidity, potentially limiting the efficiency of other photosynthetic aquatic life.[14] CyanoHABs also pose a risk to public and environmental health by producing a variety of toxic metabolites, collectively known as cyanotoxins.[13]

## 1.2 Cyanotoxins

Cyanotoxins include a range of compounds that have varied mechanisms of toxicity (Table 1.1), including hepatotoxic cyclic peptides, and neurotoxic alkaloids and amino acids. These toxins present substantial risks to public health, as well as wildlife and domesticated animals.

The potential dangers associated with cyanotoxins necessitate careful monitoring, especially during cyanobacterial blooms, to limit human exposure. Regulatory authorities worldwide, including in Australia, monitor some of these toxins, although the methods and scope of monitoring can vary. Some cyanotoxins, such as the hepatotoxic microcystins, are well understood and have defined exposure guidelines to limit risk to public health, while others such as neurotoxic amino acids are less understood.

The World Health Organization (WHO) has established provisional guidelines for some cyanotoxin concentrations in drinking water (Table 1.1),[15] some of which have been adopted by various countries. These guidelines are for the total concentration for each class of toxin (the sum of all congeners, both free and cell-bound). All WHO guideline values are provisional due to uncertainty related to congeners, such as compound specific toxicity, or limited available data.[15] Accurately determining safe concentration limits is challenging due to the complexity of analysing individual toxins and the lack of comprehensive toxicological data.

**Table 1.1** Cyanotoxin classes, toxicity and monitoring guidelines for drinking water.

| Class | Name | Effect | WHO Guideline values[15] |
|---|---|---|---|
| Cyclic peptides | Microcystins | Hepatotoxin | 1 µg/L<br>(12 µg/L for acute exposure) |
| | Nodularins | Hepatotoxin | Marine toxin-N/A to drinking water |
| Alkaloids | Anatoxin-a | Neurotoxin | Insufficient data-proposed at<br>30 µg/L for short-term exposure |
| | Homoanatoxin-a | Neurotoxin | Contributes to anatoxin-a guideline limits (Considered a congener of Anatoxin-a) |
| | Guanitoxin (also known as anatoxin-a(S)) | Neurotoxin | None |
| | Saxitoxins | Neurotoxin | 3 µg/L |
| | Aetokthonotoxin | Neurotoxin | None |
| | Cylindrosper-mopsin | Hepatotoxin, Nephrotoxin | 0.7 µg/L<br>(3 µg/L for acute exposure) |
| Lipopolysac-charides | Multiple | Irritant- less potent than others.[16] | None |
| Amino acids | BMAA | Neurotoxin | None |
| | 2,4-DAB | Neurotoxin | None |
| | AEG | Neurotoxin | None |

To simplify risk assessment for recreational waters and to not require toxin analysis, many countries have adopted the use of total cyanobacterial biomass or cell counts as an indicator of risk.[17] This is often implemented as a tiered risk alert system, such as the one utilised in Australia (Table 1.2).[18] This approach helps estimate the overall hazard of a cyanobacterial bloom without the need for complex, toxin-specific analyses. By monitoring cyanobacterial biomass, authorities can issue timely alerts and take preventive measures to protect public health from exposure via recreational waters. While this method provides a practical and cost-effective solution, it only provides limited information which often leads to under- or overestimating the health risk.[19] Alternatively, direct toxin analysis offers a more accurate assessment, but it is expensive, time-consuming, and requires specialised expertise and analytical instrumentation, making it less accessible in many cases.

Among the cyanotoxins, the amino acid β-N-methylamino-L-alanine (BMAA) is of increasing concern due to its suspected link to neurodegenerative diseases. Unlike other cyanotoxins that have well-understood mechanisms of acute toxicity, BMAA's effects are chronic, which complicates both its detection and risk assessment. Although BMAA is often classified as a cyanotoxin, its production is not exclusive to cyanobacteria. It is also known to be produced by other phytoplankton, such as diatoms and dinoflagellates.[20-22] Its low concentrations in the environment, combined with the complications of assessing chronic exposure, make routine monitoring difficult. However, because of its potential for long-term harm, there is growing interest in developing temporal monitoring strategies specifically for BMAA to better understand its environmental persistence and risks, which are still poorly understood compared to other cyanotoxins.

**Table 1.2** Australian guidelines for cyanobacterial alert levels for recreational waters. At least one of the requirements for the tier must be reached to reach that alert level.[18]

| Alert level 1- Green: Surveillance | Alert level 2- Amber: Alert | Alert level 3- Red: Action |
|---|---|---|
| Cell count:<br>- 500-5000 cells/mL of *M. aeruginosa*<br>Biovolume:<br>- 0.04-0.4mm$^3$/L total cyanobacteria | Cell count:<br>- 5000-50 000 cells/mL of *M. aeruginosa*<br>Biovolume:<br>- 0.4-4 mm$^3$/L total cyanobacteria where a known toxin producer is the dominant species<br>- 0.4-10 mm$^3$/L total cyanobacteria | Toxin concentration:<br>- ≥ 10 µg/L total microcystins<br>Cell count:<br>- 50 000 cells/mL of *M. aeruginosa*<br>Biovolume:<br>- ≥ 4 mm$^3$/L total cyanobacteria where a known toxin producer is the dominant species<br>- ≥ 10mm$^3$/L total cyanobacteria<br>Cyanobacterial scum:<br>- Consistently present |

## 1.3 BMAA

β-N-methylamino-L-alanine (BMAA) is a non-protein amino acid (NPAA) cyanotoxin that was first linked to the high incidence of amyotrophic lateral sclerosis and Parkinsonism-dementia complex (ALS-PDC) on the Pacific island of Guam.[23] It was first isolated from cycad seeds in Guam, establishing a possible exposure route through the local diet,[23, 24] and has since been found globally in aquatic and terrestrial cyanobacteria,[25, 26] diatoms,[20, 21] and dino-

flagellates,[22] as well as in higher trophic levels,[27, 28] including foods for human consumption.[29, 30] BMAA has also been detected in the brains of Alzheimer's patients,[31] and has had its neurotoxicity extensively reported.[32-37] Despite this, the role of BMAA in neurodegenerative disease remains under debate, with conflicting views on both its environmental production and its toxicity mechanisms. One point of contention is the inconsistent detection of BMAA in cyanobacteria, with some studies failing to detect the toxin,[38, 39] and others regularly reporting its presence.[40-43] Additionally, the relatively low and variable concentrations of BMAA detected in the environment are often below the levels required to elicit an acute toxic effect, further complicating the directly attributable effects.[44, 45] It has also been argued that many current toxicity studies, which primarily focus on acute exposure, fail to accurately model the chronic toxicity mechanisms that BMAA is suspected to exert.[46] While environmental concentrations may be too low to cause immediate acute effects, BMAA's chronic toxicity is poorly understood, suggesting that even low levels of BMAA should not be disregarded.

### 1.3.1 Occurrence & bioaccumulation of BMAA

BMAA is found in a range of samples and environments, as it bioaccumulates up the food chain. The bioaccumulation of BMAA was first documented in Guam,[47] where BMAA produced by cyanobacteria on the coralloid roots of cycad plants was taken up by the plant and found in higher concentrations in the seeds, which in turn were consumed by flying foxes.[48, 49] Bioaccumulation of BMAA in the food chain resulted in much higher levels of BMAA in the flying foxes than were present in the cyanobacteria or cycad seeds, with up to 10, 000x higher concentrations of BMAA in flying foxes than cyanobacteria.[48, 50] Flying foxes, along with the cycad seeds, were part of the traditional diet of the Chamorro people of Guam, establishing a pathway of exposure, and establishing a link to the high rate of ALS-PDC within the population.[24] However, the levels of BMAA in flying foxes is debated in the literature,[45, 51] as the methods used for these early studies are no longer considered suitable for the analysis of BMAA in complex sample matrices,[52] and a recent study failed to detect BMAA

in samples that were previously reported to have high concentrations.[53] This debate is further complicated by the species extinction, which prevents any new samples from being collected. As such, all studies quantifying BMAA in flying foxes have been limited to historical samples and are therefore limited in sample sizes and have the potential to be impacted by stability issues. Outside of Guam, studies have shown evidence of BMAA bioaccumulation in marine and freshwater environments, including in the United States,[27] Sweden,[54] and France.[55, 56] Many of these studies have focussed on food intended for human consumption.[29] Filter-feeding shellfish, such as mussels, clams, and oysters, are particularly effective at taking up BMAA and have therefore been relatively well studied.[29, 54, 57] BMAA has also been detected in a range of aquatic crustaceans,[30, 58, 59] mammals,[60, 61] and fish,[62, 63] increasing the potential for exposure through marine life and showing its widespread occurrence in aquatic environments. Despite being common in aquatic environments, there has been little success with detecting BMAA in natural water samples.[64, 65] However, the detection of BMAA in water has likely been limited by its occurrence in very low concentrations. The majority of the detections in studies that have successfully detected BMAA in natural water found concentrations below 2 ng/L, which is lower than the limits of detections of other studies that have attempted this analysis. [66, 67]

Research has also shown that BMAA can be transferred into terrestrial food chains, through dietary intake of either cyanobacteria or mussels.[68, 69] There is also evidence that crops irrigated with contaminated water can absorb BMAA, potentially introducing it into terrestrial food chains through plants.[70, 71] BMAA has also been detected in terrestrial cyanobacteria present in soil crusts,[72] which can produce contaminated dust when disturbed, enabling BMAA to become air-borne and potentially inhaled. [73]

Concentrations of BMAA present in different environments, regions, seasons, and food webs vary significantly, ranging from ng/g to mg/g.[74, 75] Some differences in BMAA concentrations can be attributed to temporal variations, with seasonal fluctuations or changes during bloom events.[76, 77] Other impacting

factors include environmental factors, such as nutrient availability and temperature,[78, 79] that would encourage or discourage the production of BMAA, the presence of bacteria which contain BMAA in peptides,[80, 81] as well as the dominant species or strain of cyanobacteria's ability to produce BMAA.[82] Some variation has also been attributed to different instrumental methods, with many older studies utilising analytical techniques that are no longer considered selective enough for this analysis. [52]

The wide variability of BMAA concentrations in the environment complicates the development of risk assessments and the establishment of standard monitoring practices by government authorities. Considering each of the different ways BMAA occurs in the environment is necessary for understanding potential exposure and, as much as possible, the associated risks. Establishing risk levels of BMAA is further complicated by its potential co-occurrence with other cyanotoxins, as it has been found in the environment alongside other cyanotoxins, such as microcystins or anatoxins.[83-85]

### 1.3.2 BMAA toxicity

BMAA is a neurotoxin that has hypothesised links to clusters of neurodegenerative disease through chronic exposure. These clusters are primarily in regions near water bodies prone to large cyanobacterial blooms[86-88] or that have a seafood-heavy diet.[55, 58] A further link has been proposed between the high incidence of ALS present in veterans of the Gulf War,[73] where exposure to BMAA could have occurred from military activities disturbing terrestrial cyanobacterial mats and creating aerosols, which could then be inhaled.

Since being linked to the high occurrence of ALS-PDC in Guam, BMAA has demonstrated neurotoxic effects in both *in vitro* and *in vivo* studies.[89, 90] It has also been detected in the brains of ALS-PDC patients in Guam and Alzheimer's patients in North America. In the case of ALS-PDC-related neurodegeneration, a slow progression was observed, with the onset of symptoms being delayed by several years post-exposure.[91] This suggests that BMAA acts as a chronic toxin

with potentially complex toxicity mechanisms. Recent research has also detected BMAA in the olfactory bulb, indicating that exposure through inhalation could bypass the blood-brain barrier and contribute to neurotoxicity.[92, 93] The olfactory pathway may, therefore, represent an additional route of exposure, which could be particularly relevant for populations living near cyanobacteria-affected water bodies.

Several mechanisms of BMAA toxicity have been proposed, all of which lead to neurological damage and are facilitated by its ability to cross the blood-brain barrier.[89] Some of these key mechanisms include excitotoxicity,[94, 95] gliotoxicity[96, 97] and inducing oxidative stress.[98, 99] More recently it has been suggested that the toxicity of BMAA arises from a combination of mechanisms,[100] with one study looking at sub-excitotoxic levels suggesting that its toxicity is driven by complex interactions between neuronal and glial cells, which exhibit different responses to BMAA.[101] This combination of toxicity mechanisms better explains chronic toxicity, as the concentrations used are below the levels needed to induce acute neurotoxicity.[101]

Concerns about the health impacts of BMAA are amplified by its co-occurrence with other toxins. Some cyanobacteria species that produce BMAA are also known to produce other cyanotoxins, such as microcystins, anatoxins, and saxitoxins.[40, 83, 84, 102] These combinations may have additive or synergistic effects, increasing the overall toxicity.[83, 103] BMAA also exhibits increased toxicity when present with 2,4-diaminobuytric acid (2,4-DAB), further amplifying its harmful effects compared to when these toxins are present individually.[37] Heightened neurotoxicity has also been observed when BMAA is present alongside other toxic environmental contaminants, such as methylmercury.[104] The co-occurrence of BMAA with such toxins raises further concern for its potential impact on human health.

### 1.3.3 Isomers of BMAA

BMAA is commonly found and analysed alongside two of its isomers (Figure 1.1), 2,4-DAB, and N-(2-aminoethyl)glycine (AEG), both of which also exhibit their

own neurotoxicity.[105, 106] Studies examining the mechanisms and extent of AEG's toxicity have conflicting results, with a recent finding claiming it to be the most toxic of the three isomers,[107] and others finding it to be the least.[37] More recently, studies have also included the isomer β-amino-N-methyl-alanine (BAMA) in the analysis of BMAA, primarily to ensure confidence in identification, [53, 108] as there is currently no toxicological data for this isomer.[109]

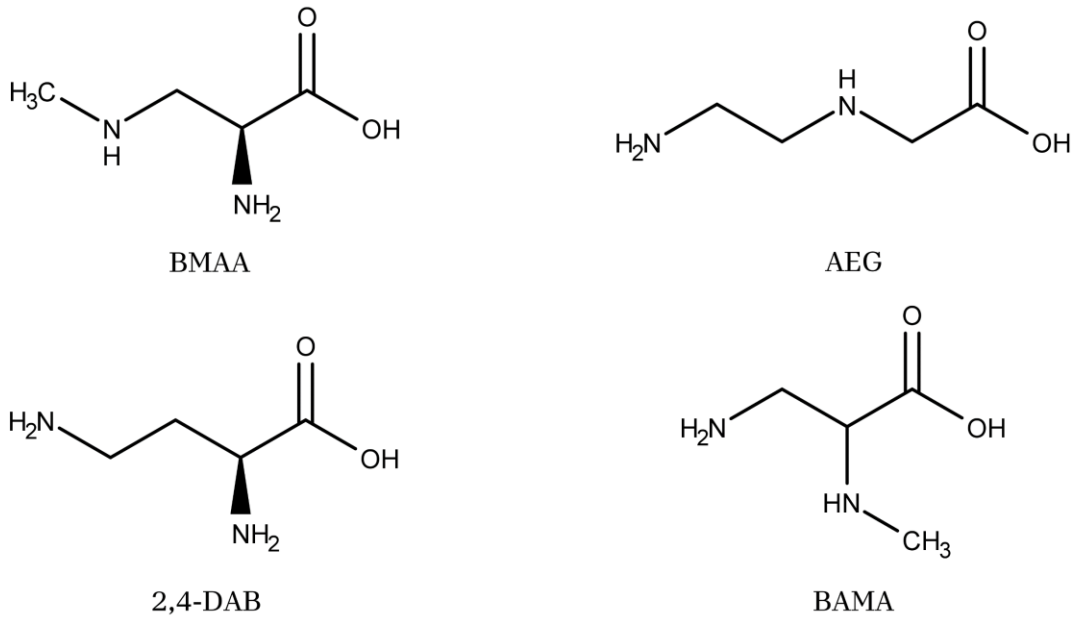


**Figure 1.1** Structures of BMAA and its common isomers AEG, 2,4-DAB and BAMA

## 1.4 Analytical methods

BMAA is typically analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). One or more isomers are often included in the analysis alongside BMAA, both to ensure confidence in BMAA detection and to better assess total toxin concentrations. Other analytical techniques have been utilised for quantifying BMAA, including liquid chromatography-fluorescence detector (LC-FLD),[25, 52] enzyme-linked immunosorbent assay (ELISA)[110] and gas chromatography-mass spectrometry (GC-MS),[111] but these have been deemed unsuitable due to low sensitivity and poor selectivity, which can lead to over- or underestimations of BMAA concentrations.[52, 82] LC-MS/MS analysis for BMAA

utilises either reverse phase liquid chromatography (RPLC) or hydrophilic interaction liquid chromatography (HILIC).[52, 112]

RPLC is a more widely used technique as its use is well established and mechanisms of retention understood. Retention in RPLC is reliant upon hydrophobic interactions between the analyte and the stationary phase, making it highly suitable for non-polar analytes. Conversely, HILIC employs newer chemistries for the separation of more polar analytes, utilising retention mechanisms that are well-researched but not yet fully understood. [113] As BMAA is a polar amino acid, it is highly suitable for separation by HILIC. However, there are drawbacks of using this technique, such as increased run costs, reliance on highly buffered systems, poor repeatability and long equilibration times. There are also some benefits, such as better desolvation in ESI due to the use of a highly organic mobile phase and its ability to separate compounds that can't be retained on RPLC stationary phases, which includes BMAA and its isomers.

For BMAA to be analysed by RPLC, derivatisation is required. Derivatisation is a selective chemical alteration that can improve chromatographic separation by introducing functional groups to the analyte, which either increase or decrease analyte polarity and, therefore, alter its retention on the stationary phase.[114] Derivatisation can also improve MS sensitivity by enhancing ionisation in the ESI and increasing the analyte's mass, moving the compound out of the noisy, low *m/z* range. However, derivatisation often involves hazardous materials and adds additional steps to sample preparation workflows.

There are multiple different, well-validated derivatisation approaches,[52, 74, 82, 115-119] with both commercial kits and methods developed in-lab being regularly used. The most common derivatisation method for BMAA analysis is the AccQ-Tag Ultra derivatization kit by Waters, which uses 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). Other methods include using either propyl chloroformate (PCF),[26, 40, 111] 9-fluorenylmethyl chloroformate (FMOC-Cl)[66] and dansyl chloride (DNS)[118, 120] (Figure 1.2).

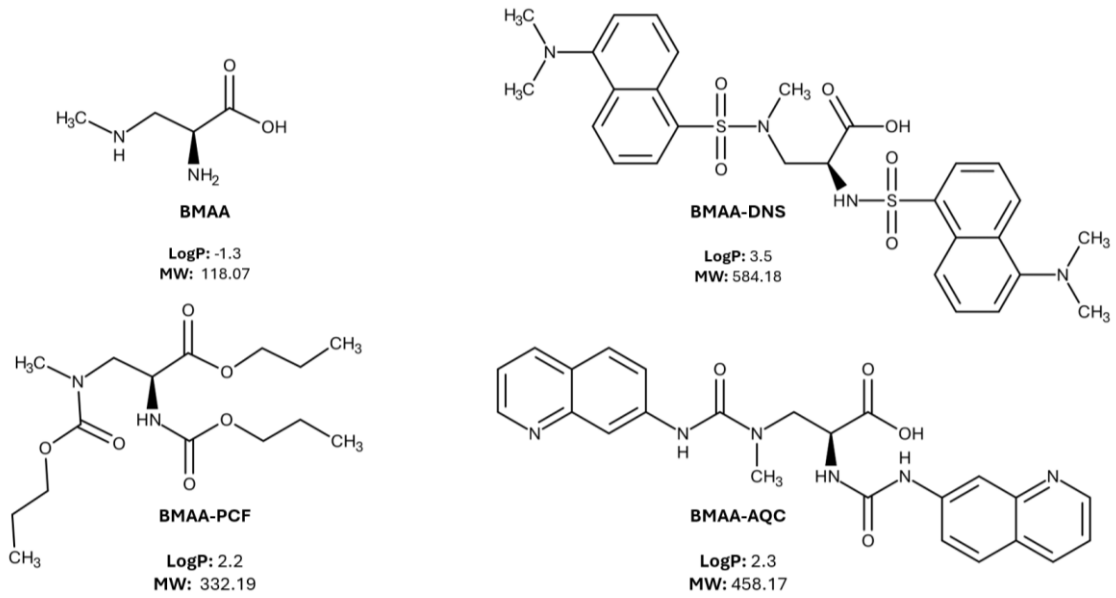


**Figure 1.2** Structures and logP values of BMAA and BMAA derivatised with the most common derivatising agents, propyl chloroformate (PCF), dansyl chloride (DNS), and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). LogP was estimated using Chemaxon MarvinSketch MW is the exact mono-isotopic mass.

Derivatisation methods are typically considered to be more sensitive than the underivatised HILIC methods, with the exception of a comparison study by Faassen et al.[52] who found lower limits of detection (LODs) in sample matrices with their HILIC method. This study attributes the higher LODs in derivatised samples to be a result of dilutions during derivatisation. However, dilution is not a feature in all derivatisation methods, with some extraction methods even allowing for preconcentration during sample clean-up and derivatisation.[21, 40] This work also found that derivatisation and RPLC were more sensitive for standards, further suggesting that derivatisation methods improve sensitivity when any necessary dilutions are accounted for. A recent review of the analytical methods for quantification of BMAA found that many derivatised RPLC methods had up to 40x improvements in sensitivity over underivatised HILIC methods.[82]

When analysing BMAA, most studies utilise triple quadrupole mass spectrometry (QQQ), due to its ability to confidently differentiate isomers through unique multiple reaction monitoring (MRM) transitions or transition ratios. While high-resolution mass spectrometry (HRMS) can be useful for

untargeted analysis, it has lower sensitivity and is not necessary for targeted BMAA analyses, where isomer differentiation is the primary concern. However, HRMS is still used in some studies and can be useful when other analytes, such as other cyanotoxins, are included in the methods.[121]

Despite the sensitivity of LC-MS/MS for detecting BMAA, there is a notable lack of consistency in results across different analytical methods. For example, a review of BMAA analyses in cyanobacteria found that 92% of samples analysed using RPLC detected BMAA, while only 57% showed detection with HILIC.[82] These inconsistencies become especially evident in comparative studies, where identical samples analysed by different LC-MS/MS techniques found varying BMAA concentrations. [52, 112] Incomplete method validation, including inadequate reporting of detection limits and spike recoveries, coupled with the inconsistencies between analytical techniques have often led to a lack of confidence in both positive detections and non-detects of BMAA.[52, 122]

Analytical methods have also been limited to detecting BMAA in cyanobacterial scum analysis (or analysis of higher trophic levels) rather than directly in water. This presents a range of issues as it is not an accurate measure of water risk or toxicity. Analysis of scum samples and the species within it doesn't account for any deeper, planktonic BMAA producing cyanobacteria, and it fails to account for concentrations of toxins that have been released from the cyanobacteria cells into the water. This is a known transport mechanism for other cyanotoxins, such as microcystins, wherein the toxin is released from the cell and into the water column upon cell death as the bloom progresses.[123, 124] Whilst important in establishing the presence of BMAA and the species that could be responsible for its production, scum sampling is not comprehensive and can misrepresent total toxicity and exposure risks when used as representative of a waterway.

Attempts at analysing water samples have been hampered by issues including very low concentrations and lack of affinity with typical preconcentration methods, as the toxin's high polarity excludes the use of standard reverse-phase extraction cartridges. The pre-concentration methods that have been

developed to mitigate the issues of low water concentrations have shown limited success in the detection of BMAA in spiked samples.[64, 65] To date, few have determined the presence of BMAA in natural waters.[66, 67]

The variation of BMAA detection across different analytical methods hinders the development of a standardised analysis protocol for government agencies and water bodies. Additionally, the current methods of scum sampling and low sensitivity of water analysis may underestimate the risk and provide few insights into variations of toxin levels over time, that could inform risk levels for chronic exposure. Therefore, a more comprehensive analysis method that provides temporal information is required.

## 1.5 Bioindicators

One approach to achieving temporal pollution and contaminant monitoring is by using sentinel animals as bioindicators.[125] While the simplest way to test water pollution is through direct water analysis, water pollutants can become heavily diluted in large bodies of water and require additional sample preparation to pre-concentrate the analytes of interest.[126] Sampling of water also has the limitation of indicating pollutant levels at a single time point, requiring regular sampling for longer-term monitoring. Contaminant levels in water can also be assessed by measuring the concentrations of contaminants accumulated in local biota.[125] This is especially true for persistent pollutants, which are effective at accumulating in the environment and can even be biomagnified,[127] a process in which concentrations are increased in higher trophic levels. The accumulation of pollutants into organisms enables bioindicators to be used as a proxy for environmental contamination. Bioindicators can be a range of organisms, including fish, shellfish, and plants.[125]

## 1.6 Mussels as bioindicators

Many bioindicator studies utilise filter-feeding bivalves, such as mussels, oysters and clams, as they have unique features that make them ideal for this work.[128] Bivalves are typically sedentary, robust, long-lived and widespread creatures that can concentrate many pollutants that are present in water and plankton. [129, 130] Early bioindicator studies focussed on contaminants in marine environments, including the highly successful and long running Mussel Watch program in the United States,[131, 132] that now involves more than 140 different pollutants. Many of the attributes that made marine bivalves suitable for long-running bioaccumulation studies are applicable to their freshwater and estuarine counterparts. Initial freshwater studies assessed bivalve viability for monitoring metals and pesticides but have since expanded to a greater range of pollutants. In comparative studies between the viability of freshwater and marine bivalves as bioindicators, similar results were seen. [133]

Since the early marine and freshwater studies, mussels have had widespread use over several different fields. They are commonly used for tracking heavy metals,[134] microplastics,[135] pharmaceuticals[136] and industrial pollutants.[137] Less work has been done on cyanotoxins. However, many of the commonly studied cyanotoxins have been detected and monitored by mussels, typically in estuarine and marine waters, including microcystins,[138, 139] anatoxins,[140] and nodularin.[141]

## 1.7 BMAA in mussels

Whilst there is limited work into the use of mussels of bioindicators of BMAA, the suitability for mussels for environmental monitoring is supported by the regular detection of BMAA in bivalves. These include the marine blue mussel genus *Mytilus*, which are often used as bioindicators of other toxins and pollutants[137] and regularly have high concentrations of BMAA resulting in their use as a positive control in some BMAA analyses.[53, 142] Additionally, studies have shown BMAA biomagnification throughout various food webs, in which it

makes its way up trophic levels.[23, 143, 144] As mussels filter feed and much of their diet is composed of phytoplanktonic organisms, they would ingest and accumulate BMAA from its source, as well as any present in the water that is filtered.

Recent work has shown that mussels may be useful as bioindicators of BMAA.[145, 146] In these studies mussels were shown to take up free BMAA dissolved in the tank water. However, this work did not demonstrate the bioavailability of BMAA from cyanobacteria in either controlled lab experiments or *in situ*. By working with dissolved BMAA, these studies act as a proof of concept, but further work needs to be done to assess the uptake from phytoplankton and environmental waters. These studies also did not test for BMAA's isomers which contribute to total toxicity.

Mussels have the potential for high exposure to BMAA through filtering water and digesting phytoplankton. Their capacity to bioconcentrate could make them a suitable solution to the need for long-term monitoring indicative of a chronic exposure model that considers changing concentrations over short periods of time and total toxin concentrations to give a more accurate overview of the risk associated with a cyanobacterial bloom.

## 1.8 Thesis Aims:

There are multiple difficulties with analysing BMAA in the environment, which hinders the ability for regular monitoring practices to be taken up by government authorities and, therefore, put into place controls to reduce risk. Current issues include difficulties with analysis in trace concentrations as well as monitoring practices that are suitable for temporal assessments, which are necessary to account for the risk of chronic exposure to BMAA. This thesis aims to address these issues by assessing the viability of the freshwater mussel *Velesunio ambiguus* as a bioindicator of BMAA concentrations over time. The specific aims of this thesis were to:

- Investigate temporal dynamics of BMAA in the environment by monitoring concentrations over the progression of a bloom event
- Compare analytical approaches to BMAA analysis and develop a sensitive and selective method for analysing BMAA in mussel matrix
- Determine the uptake and depuration dynamics of BMAA by *V. ambiguus* exposed to controlled amounts of BMAA-producing cyanobacteria
- Assess the suitability of *V. ambiguus* as a bioindicator of BMAA *in situ* by deploying mussels into environmental waters.

# Chapter 2: Temporal dynamics of BMAA concentrations in cyanobacteria

## Chapter overview

The neurotoxin BMAA is typically analysed through single samples of cyanobacterial blooms. However, its chronic toxicity mechanisms require a more comprehensive, temporal approach to risk assessment. The following paper forms the foundation for this thesis by establishing the highly dynamic nature of BMAA concentrations in environmental cyanobacterial blooms. This highlights the limitations of single-time-point sampling strategies in assessing the overall risk of a cyanobacterial bloom, and justifies the need for developing long-term, temporal monitoring solutions, such as bioindicators, that are able to more accurately determine exposure risk.

## Certificate of authorship

The following chapter is published in *Molecules*.
I, Siobhan Peters, certify that the work in the following chapter has not been submitted as part of any other documents required for a degree.

Authorship contributions

| Author | Contributions | Signature |
|---|---|---|
| Siobhan J. Peters | methodology, validation, formal analysis, investigation, conceptualisation, writing-original draft, visualisation. | Production Note: Signature removed prior to publication. |
| Kenneth J. Rodgers | conceptualisation, writing- review & editing, funding acquisition. | Production Note: Signature removed prior to publication. |
| Simon M. Mitrovic | conceptualisation, writing- review & editing, funding acquisition. | Production Note: Signature removed prior to publication. |
| David P. Bishop | conceptualisation, writing- review & editing, supervision, funding acquisition | Production Note: Signature removed prior to publication. |

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# The Changes in Cyanobacterial Concentration of β-Methylamino-L-Alanine during a Bloom Event

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## Abstract:

β-N-methylamino L-alanine (BMAA) is a neurotoxin linked to high incidences of neurodegenerative disease. The toxin, along with two of its common isomers, 2,4-diaminobuytric acid (2,4-DAB) and N-(2-aminoethyl)glycine (AEG), is produced by multiple genera of cyanobacteria worldwide. Whilst there are many reports of locations and species of cyanobacteria associated with the production of BMAA during a bloom, there is a lack of information tracking changes in concentration across a single bloom event. This study aimed to measure the concentrations of BMAA and its isomers through the progression and end of a cyanobacteria bloom event using liquid chromatography-triple quadrupole-mass spectrometry. BMAA was detected in all samples analysed, with a decreasing trend observed as the bloom progressed. BMAA's isomers were also detected in all samples, however, they did not follow the same decreasing pattern. This study highlights the potential for current sampling protocols that measure a single time point as representative of a bloom's overall toxin content to underestimate BMAA concentration during a bloom event.

## 2.1 Introduction

Cyanobacteria, often referred to as blue-green algae, are phototrophic microorganisms that are widespread in aquatic and terrestrial environments.[1] While they are a natural part of many ecosystems, certain conditions, such as eutrophication, warmer temperatures,[7] and decreased river flow rates,[9] can increase growth and result in blooms.[13] Cyanobacterial blooms are increasing in prevalence and intensity primarily due to human activities contributing to improved growth conditions, including agricultural runoff, water extraction for irrigation, and rising global temperatures.[10]

Cyanobacterial blooms can be a public health hazard because of the range and potency of the toxins that they produce.[147] These toxins, collectively known as cyanotoxins, include the neurotoxin β-N-methylamino L-alanine (BMAA). BMAA is a non-protein amino acid (NPAA) that became of interest after it was linked to the high incidence of amyotrophic lateral sclerosis and Parkinsonism-dementia complex (ALS-PDC) on the Pacific island of Guam.[23] BMAA was first isolated from cycad seeds in Guam, establishing a possible exposure route through the local diet.[24] It was later shown to be produced by symbiotic cyanobacteria in the coralloid roots of the cycads and to bioaccumulate up the food chain and make its way into the native food sources.[23] BMAA has since been found in cyanobacteria and diatoms globally[21, 25] and in higher trophic levels,[27, 28] including foods for human consumption,[54] and the brains of Alzheimer's patients,[31] with its neurotoxicity extensively reported in cell and animal models.[32-35, 37] Despite this, there is still much debate on the toxicity mechanisms of BMAA, with many proposed neurotoxic properties but no clearly established mechanism for chronic toxicity.[109]

Many Australian waterways are prone to developing conducive conditions for cyanobacterial growth, which can result in persistent and far-reaching blooms.[148-150] Regular, large-scale blooms present a health concern to surrounding communities, necessitating comprehensive monitoring of cyanobacteria and the toxins they produce to limit human exposure.

Consequently, several cyanotoxins, such as hepatotoxic microcystins and neurotoxic anatoxins, are regularly monitored by government authorities globally.[151] However, this does not yet extend to BMAA or the two isomers it is often found alongside, 2,4-diaminobuytric acid (2,4-DAB) and N-(2-aminoethyl)glycine (AEG). In addition to exhibiting its own neurotoxicity, 2,4-DAB contributes to combined neurotoxicity when present alongside BMAA.[37] Studies examining the toxicity of AEG have reported conflicting results, with a recent report claiming it to be the most toxic of the three isomers[107] and others suggesting it to be the least toxic.[37]

Previous studies have found BMAA and 2,4-DAB in a range of cyanobacterial bloom samples, including studies focusing on their presence in Australian blooms and cyanobacterial cultures.[26, 40] However, to date, no study has assessed the concentrations of these toxic NPAAs across multiple time points during a single bloom event. Knowledge of the changes in concentration of BMAA and its isomers within a bloom over time has the potential to aid in recognising periods of increased risk of human exposure and may contribute to understanding the significance of toxin production to cyanobacteria. This study used liquid chromatography-triple quadrupole-mass spectrometry (LC-MS/MS) to measure the concentrations of BMAA and its isomers over time to track changes in toxin concentration during the progression of a bloom event.

## 2.2 Results

The concentrations of BMAA, 2,4-DAB, and AEG were determined for each sample using LC-MS/MS. The ‘free’ samples refer to the non-hydrolysed soluble fraction obtained following protein precipitation with 10% TCA. The second fraction was the hydrolysed precipitate produced by the addition of 10% TCA referred to as the ‘insoluble’ fraction. BMAA, 2,4-DAB, and AEG were quantified in two fractions from all four samples, over three different time points, with the final two samples being collected on the same day but selectively sampled with the ‘intact’ sample consisting of mostly alive cells, and the ‘decayed’ sample consisting of deteriorated scum.

Concentrations of BMAA in both fractions decreased as time progressed (Figure 2.1). The insoluble protein fraction had the highest concentration of BMAA across all samples, ranging from 5.7 ± 4 ng $g^{-1}$ dw to 21 ± 6 ng $g^{-1}$ dw (Table 2.1). There was little difference between the intact and decayed sample concentrations sampled from the final time period in both fractions (Figure 2.2).

2,4-DAB was only quantified in the insoluble fraction and was below the limit of quantification in the free fraction. The 2,4-DAB results from the insoluble fraction did not follow the same trend of a decreasing concentration over time as was seen in the BMAA insoluble fraction (Supplementary Figure 2.2), as 2,4-DAB concentrations peaked on the second sampling day before dropping down to concentrations lower than the first sampling point.

AEG was detected in both fractions from all dates, with an initial decrease and then a sharp increase in concentration in the insoluble fraction (Supplementary Figure 2.3). The free fraction levels were relatively consistent across all time points over the collection period (Table 2.1).

**Table 2.1** Toxin concentrations ± standard deviation (n = 3) in environmental samples. <LOQ denotes detections below the limit of quantification

| | BMAA (ng $g^{-1}$) | | 2,4-DAB (ng $g^{-1}$) | | AEG (ng $g^{-1}$) | |
|---|---|---|---|---|---|---|
| Date | Free | Insoluble | Free | Insoluble | Free | Insoluble |
| 29 January | 7.01 ± 0.03 | 20.8 ± 5.5 | <LOQ | 524 ± 105 | 5.16 ± 1.36 | 31.8 ± 2.7 |
| 2 February | 6.96 ± 0.01 | 9.49 ± 0.76 | <LOQ | 533 ± 137 | 4.65 ± 0.93 | 13.2 ± 5.6 |
| 7 February (intact) | <LOQ | 5.65 ± 0.39 | <LOQ | 221 ± 50 | 4.87 ± 0.48 | 134 ± 47 |
| 7 February (decayed) | <LOQ | 5.65 ± 0.22 | <LOQ | 340 ± 18 | 3.91 ± 0.98 | 86.0 ± 22.2 |

BMAA, 2,4-DAB, and AEG concentrations in the decayed (Table 2.1) intact cell samples taken from the final time point were compared (Figure 2.2). Whilst the BMAA concentrations showed little difference between the two samples, higher concentrations of 2,4-DAB and lower concentrations of AEG were found in the decayed sample.

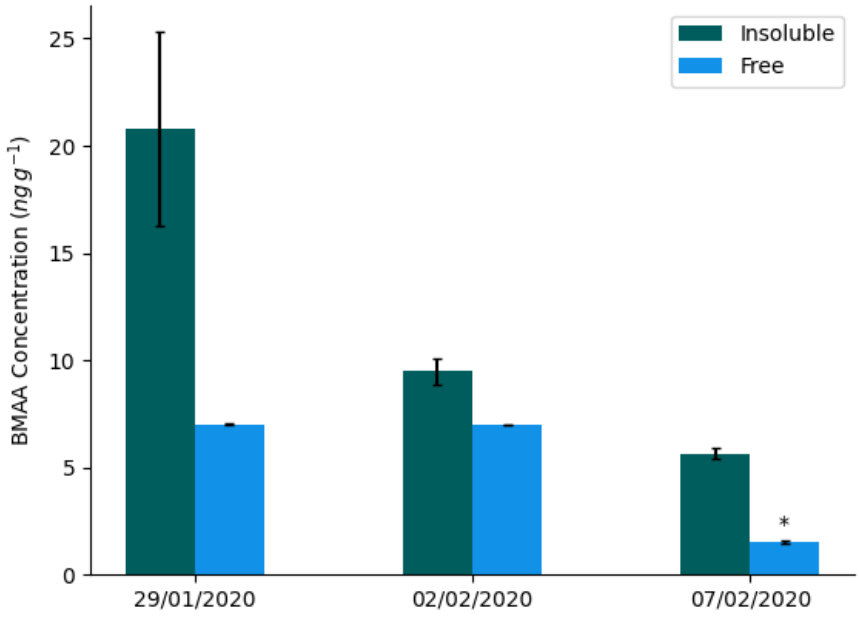



**Figure 2.1** BMAA concentrations ± standard deviation in environmental cyanobacteria samples (n = 3, n = 6 for 7 February with combined decayed and the intact results). * Denotes an estimation of concentration, as this sample was detected, but below the LOQ

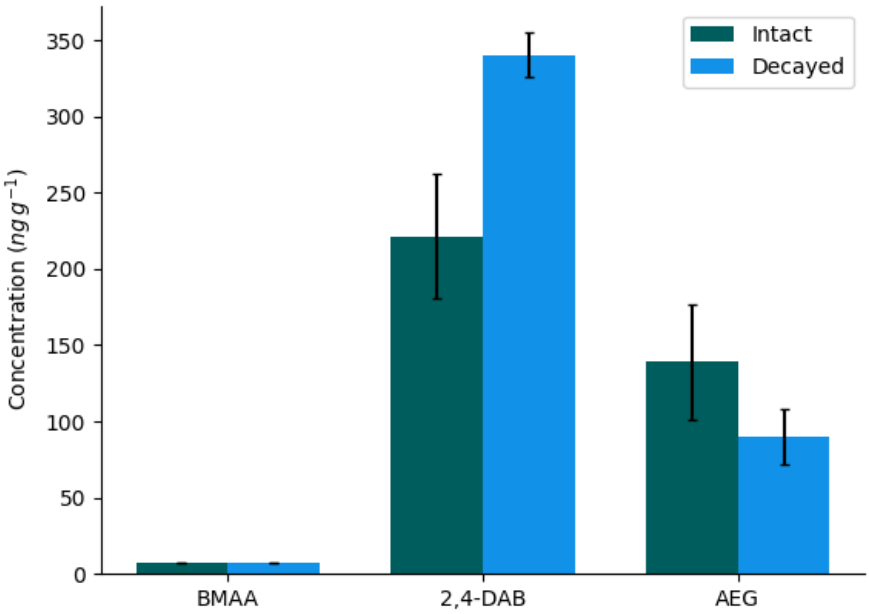



**Figure 2.2** Total BMAA, 2,4-DAB and AEG concentrations ± standard deviation in samples collected on 7 February 2020 from the intact scum and the selectively sampled decaying scum (n = 3)

## 2.3 Discussion

Many Australian cyanobacteria, including the bloom forming species of this study (*Dolichospermum crassum),* are known to produce BMAA and its isomers.[26, 40] Some Australian cyanobacteria can also produce other toxins such as hepatotoxic microcystins, and neurotoxic saxitoxins[148] and anatoxins[152]. When assessing risk, it is essential to account for changes in cyanotoxin production during different periods of bloom development and collapse. Despite this, there has been no work to date on monitoring changing concentrations of BMAA and its isomers within a bloom event. *Dolichospermum crassum* has had both positive, and negative BMAA results reported both within Australia[40] and internationally.[43, 111] Considerable variation in toxin concentration within a single study and sampled from nearby locations have also been documented.[111] There has been little discussion about the impacts of these differences or why they occur. In the study conducted by Esterhuizen and Downing[111] 0.56 µg $g^{-1}$ of BMAA was present in one isolated culture of

*Dolichospermum*, whilst no BMAA was found in another. Similar discrepancies were also found in cultures of other genera in the same study. The variations seen in these studies have not yet been tracked over a period within the same bloom event. However, the variance in these previous studies' results is consistent with the results of this study, in which significant differences in BMAA concentrations occurred within samples taken from the same site and bloom event across ten days. The present study's large changes in concentration across samples taken from the same bloom but across different days highlight the problematic nature of using a single sample point as a representative result.

The previous failure to detect BMAA in some *Dolichospermum* samples[40, 43, 111] could result from method limitations,[52] genetic factors, or conditional toxin production.[62, 79]Although the role of BMAA in cyanobacteria is not fully understood, some factors that impact the production of BMAA have been explored. Environmental factors have been shown to influence BMAA production, and several studies have described how nutrient availability affects both cyanobacteria proliferation[153] and toxin production.[154, 155] Nitrogen starvation has been linked to increased BMAA production in *Microcystis* sp., with nitrogen reintroduction causing a decrease in toxin levels.[78] Further, studies using the same genus of cyanobacteria have shown that high nitrogen environments correspond with stimulated cyanobacteria growth.[153] Increased growth, whilst not directly related to toxin production, is still of concern as it can facilitate the development of cyanobacterial blooms and increase exposure.

Alongside the toxin production dynamics, variances in cyanotoxin concentration for the better-studied microcystins have been associated with the release of intracellular toxins into the water column or growth media upon cell death.[123, 124] These studies are in contrast with the results of the current study that shows decreasing BMAA content during bloom senescence, as the microcystin concentration in the water column increased as the bloom progressed.[123, 124, 156] However, this is looking at the amount of toxin released, rather than the content remaining in the cyanobacteria scum. These

microcystin analyses also required solid phase extraction (SPE) for clean-up and pre-concentration of the water samples. The analysis of BMAA is typically restricted to environmental cyanobacteria samples or laboratory culture isolates, limiting the capacity to determine if it also undergoes intracellular release into surrounding water.

There are currently several reports of the viability of SPE for the preconcentration and clean-up of water samples,[64, 65] yet relatively few have successfully detected BMAA in unspiked, natural waters.[66, 67] This creates a difficulty in analysing BMAA in water samples and complicates tracking the toxin from the intra- to the extracellular and the potential release of BMAA into the water column. This, therefore, limits comparisons between the drop in concentration of BMAA over the bloom lifecycle and the increase of microcystins under similar circumstances. The release of BMAA into the water column is of notable concern as it would potentially increase routes of exposure. High concentrations in water increase the likelihood of toxin aerosolisation[157] and the uptake of BMAA by plants,[70] two pathways hypothesised to result in human exposure to BMAA.[70]

The similarity in BMAA concentrations between the decayed and the intact sample taken on the same date suggests that in this circumstance, a mechanism other than release into the water column is responsible for the decrease in concentration. If lysed cells released BMAA into the water column, there should be less in the decayed sample than in the mostly intact sample. An alternative mechanism is additionally supported by the different changes in the concentrations of the isomers. AEG changed inconsistently, with its lowest concentration being in the middle of the sampling period, whilst 2,4-DAB concentrations decreased differently to BMAA. 2,4-DAB was the only isomer to show a difference in concentration between the decayed sample and the intact sample taken from the same date. This may be related to the presence of 2,4-DAB in high concentrations in bacterial cultures,[158, 159] which may be attracted to the more broken-down cyanobacteria scum from the decayed sample.

It is also important to note that the method followed in the present study included thawing frozen algal scum. This was done prior to centrifuging and the residual supernatant was discarded. Whilst retaining the pellet formed from centrifugation and discarding the supernatant is accepted general practice for BMAA extraction from cyanobacteria for both environmental samples and laboratory cultures, it could have impacts on the analysis of soluble toxins.[26, 111, 160] The process of freeze–thawing would result in some cell lysis [161] and it is possible that soluble NPAAs were released into the discarded supernatant. Freeze–thawing was limited to a single cycle and all samples were treated identically. Despite the potential for some loss of soluble NPAAs, this would not have influenced the insoluble fraction, in which the largest changes of BMAA concentration were observed. Additionally, changes were still observed in the free fraction, suggesting that regardless of any loss during sample preparation, the concentration of toxins across samples is still inconsistent. Future studies should aim to prepare the cyanobacteria pellet prior to freezing where possible, and analyse the supernatant using SPE for sample clean-up and preconcentration to mitigate these potential issues. Varying volumes of water are required for the current SPE methods, with one suggesting that 50 mL is the ideal volume for the best recoveries,[64] and another requiring 1 L.[67] This highlights the need for considered sampling to ensure that enough sample volume is obtained to complete these analyses.

The responsive nature of the current study's sampling regime to a bloom event and the small sample set present some limitations with this work. Limited environmental data was taken at the time of the bloom, and sampling was done inconsistently throughout, leaving an incomplete picture of the total toxicity. Between 500 mL and 1.5 L of scum sample were provided at each sampling time point, and were sub-sampled from this large volume, however these are not true technical replicates.[162] Additionally, sampling was only conducted towards the end of the bloom, with no samples provided from earlier in the growth phase. Further studies are required to establish a trend between BMAA concentration and the progression of a bloom event using technical replicates

at an increased number of timepoints from the start of a bloom until cell senescence. This would allow for confirmation of the links the present study suggests, or to assess what other factors could have been affecting the changing concentrations that were seen here. Environmental monitoring and analyses of waters from the same site should also be completed alongside scum analyses. Factors such as temperature,[12, 163-165] nutrient load,[12, 164, 166] light,[164, 165] and cyanobacteria growth patterns[12, 163, 166] are associated with varying rates of microcystin production, and whilst many of these factors are currently unexplored regarding their impact to BMAA production, they could also play a role. These factors need to be considered in order to establish consistent and thorough monitoring. As it currently stands, BMAA sampling is typically a one-off grab sample, which, regardless of factors impacting the decline, this study has highlighted as being problematic for establishing a complete picture of toxicity.

Despite the limitations of this study, we have shown the concentration of BMAA changing throughout a cyanobacteria bloom event. This may provide some insight on the varying concentrations measured from the same genus in previous studies. [40, 43, 111] Our results show considerable differences in concentration over relatively short periods and suggest multiple samples need to be taken over time to properly characterise the BMAA, 2,4-DAB and AEG concentrations during bloom events.

## 2.4 Materials and methods

### 2.4.1 Cyanobacteria samples

Freshwater cyanobacterial samples were collected by Goulburn Valley Water (GVW) from a bloom event, with four samples taken on 3 occasions over ten days from 29th January to 7th February in the summer of 2020. Surface samples were collected from an untreated irrigation supply channel in inland Victoria, Australia. This supply channel is not used for human consumption or recreational use. The samples were collected by GVW due to the proximity to the treatment offshoot stream and were non-routine unscheduled sampling, as

the bloom did not impact the extraction point. The 'decayed' sample was selectively sampled on the final date from parts of the bloom that had turned blue, indicating senescence and cell death. The dominant species of all samples, except the decayed sample, were identified by GVW as *Dolichospermum crassum*. The decayed sample was unable to be identified due to cellular stresses and viscosity of the sample (Supplementary Figure 1). Samples were delivered to the laboratory on ice and then stored at -20°C until prepared for analysis.

### 2.4.2 Chemicals & standards

Analytical-grade standards were used with L-BMAA hydrochloride (BMAA HCl, ≥97%) 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%) from Toronto Research Chemicals Inc. (North York, ON, Canada), and the internal standard D-2,4-diaminobutyric-2,3,3,4,4-d5 acid dihydrochloride (d5-DAB 2HCl, ≥66%) from CDN Isotopes (Pointe-Claire, Quebec, Canada). Sample preparation agents trichloroacetic acid (TCA, ≥99.5%), acetone (≥ 99.9%) and hydrochloric acid (HCl, 37%) were purchased from Sigma-Aldrich (Castle Hill, NSW, Sydney). Chromatographic mobile phases were prepared using LC-MS grade methanol, buffered with LC-MS grade formic acid, both from Sigma-Aldrich (Castle Hill, NSW, Australia).

### 2.4.3 Sample preparation

From each time point sample, subsamples were prepared in triplicate for analysis as described by Main et al.[26] with minor modifications (described below).

#### 2.4.3.1 Cell lysis

The cyanobacterial samples were thawed and centrifuged into a pellet for 20 min (Gyrozen, 1580R Centrifuge) at 3000×*g*. The supernatant was discarded, and the remaining pellet was freeze-dried overnight or until completely dry at 0.1 mbar and -80°C to sublimate any remaining liquid. 20 mg of the dried pellet

was transferred into a 15 mL Falcon tube, and 1 mL of 10% TCA was added to the tubes along with 20 µL of the internal standard d5-DAB (1 µg $mL^{-1}$). Samples then underwent probe sonication (Sonics & Materials Vibra Cell, VC50T 50 W Ultrasonic Processor) twice for 1.5 minutes at 70% power, with the tubes left on ice between repeats, to lyse the cells. Samples were then left at 4°C overnight to allow for precipitation. Lysed cells were centrifuged at 3000×*g* for 20 minutes, with the subsequent supernatant transferred into a 2 mL tube. The remaining pellet was washed with 200 µL 10% TCA in water once before being transferred to a glass shell vial labelled 'insoluble fraction' by two washes of 200 µL 10% TCA in acetone. The subsequent supernatants from each wash were combined with the original supernatant in a tube labelled 'free fraction.'

The free fraction was dried in a centrifugal evaporator (Thermo Fisher Scientific, Savant DNA 120 Speedvac concentrator) for 24 hours and then freeze-dried at 0.1 mbar to remove all liquids. This fraction was then reconstituted in 400 µL of 20 mM hydrochloric acid.

#### 2.4.3.2 Hydrolysis

Shell vials containing the insoluble fraction pellets were placed in a centrifugal evaporator to remove any remaining liquid. Once dried, the shell vials were placed into a vacuum hydrolysis vial with 1 mL of 6 M HCl. Oxygen was removed from the hydrolysis vial using a vacuum pump to reduce pressure to 300 mbar and then refilled with nitrogen gas. This was repeated three times to reduce oxygen levels in the vial. The hydrolysis vial was then left in an oven at 110°C for 16 hours to undergo hydrolysis with gaseous HCl. Pressure built up in the vial was released once removed from the oven, and the vials were left briefly to cool. The pellets were reconstituted in 380 µL of 20 mM hydrochloric acid and 20 µL $d_5$-DAB (1 µg $mL^{-1}$). Both fractions were then transferred to 0.2 µm membrane filter tubes (Ultrafree-MC LG Centrifugal 0.2 µL PTFE Membrane Filter) and centrifuged at 5000×*g* for 30 min. All extracts were stored at −20°C until derivatisation.

#### 2.4.3.3 Derivatisation

Samples were derivatised by propyl chloroformate (PCF) using the Phenomenex® EZ: Faast™ amino acid analysis kit (Phenomenex, Sydney, NSW, Australia) before analysis by LC-MS/MS. 200 µL of each fraction underwent dispersive SPE (dSPE) using EZ: Faast™ sorbent tips, derivatisation using a PCF derivatisation agent, and liquid-liquid extraction as per manufacturer's instructions. The derivatised samples were then dried in a centrifugal evaporator before reconstitution in 50 µL of the starting mobile phase (55% methanol, 45% water).

### 2.4.4 Sample analysis

The LC-MS/MS method used for the analysis of PCF-derivatised BMAA was developed and validated by Main et al.[26] and optimised on a Shimadzu Nexera UC UHPLC coupled to a Shimadzu LCMS-8060 triple quadrupole mass spectrometer. Chromatography utilised a Kinetex C18 column (Phenomenex; 1.7µm x 2.1 x 150mm) at a column oven temperature of 35 °C with a flow rate of 0.25 mL $min^{-1}$. The mobile phase gradient started at 45% ultrapure water with 0.1% formic acid (solvent A) and 55% methanol with 0.1% formic acid (solvent B). It was raised to 68% solvent B over a 10 min gradient, then increased to 100% solvent B, which was held for 5 min, before returning to starting conditions for 2 min for equilibration. Samples were prepared in triplicate, with each replicate undergoing triplicate 1 µL injections for analysis.

An 8-point calibration curve was formed using triplicate 1 µL injections of each standard over a concentration range of 0.1 - 50 ng $mL^{-1}$. The MS/MS source was set to 4 kV with a temperature of 300°C. Nebulising and drying gas flows were set to 2 L $min^{-1}$ and 10 L $min^{-1,}$ respectively with the heating block and desolvation line at 400°C and 250°C. The MS/MS was run in MRM mode for the analysis using the $[M + H]^+$ transition, and parameters listed in Table 2.2. Shimadzu LabSolutions software was used for data analysis. A representative chromatogram is shown in Figure 2.3.

**Table 2.2** MRM and retention parameters for detection of the PCF-derivatised BMAA, 2-4 DAB, and AEG. * denotes ion transition used for quantification

| Compound | Retention Time (min) | Transitions *(m/z)* | | Collision Energy (eV) |
|---|---|---|---|---|
| 2,4-DAB | 7.3 | 333.1 → | 273.1 | -20 |
| | | | 231.1* | -12 |
| | | | 142.1 | -28 |
| d5-DAB | 7.3 | 336 .1→ | 276.1* | -10 |
| | | | 190.1 | -17 |
| | | | 102.1 | -28 |
| AEG | 8.3 | 333.1→ | 187.1* | -16 |
| | | | 99.1 | -27 |
| | | | 88.1 | -30 |
| BMAA | 8.9 | 333.1→ | 187.1* | -16 |
| | | | 159.1 | -20 |
| | | | 73.1 | -30 |

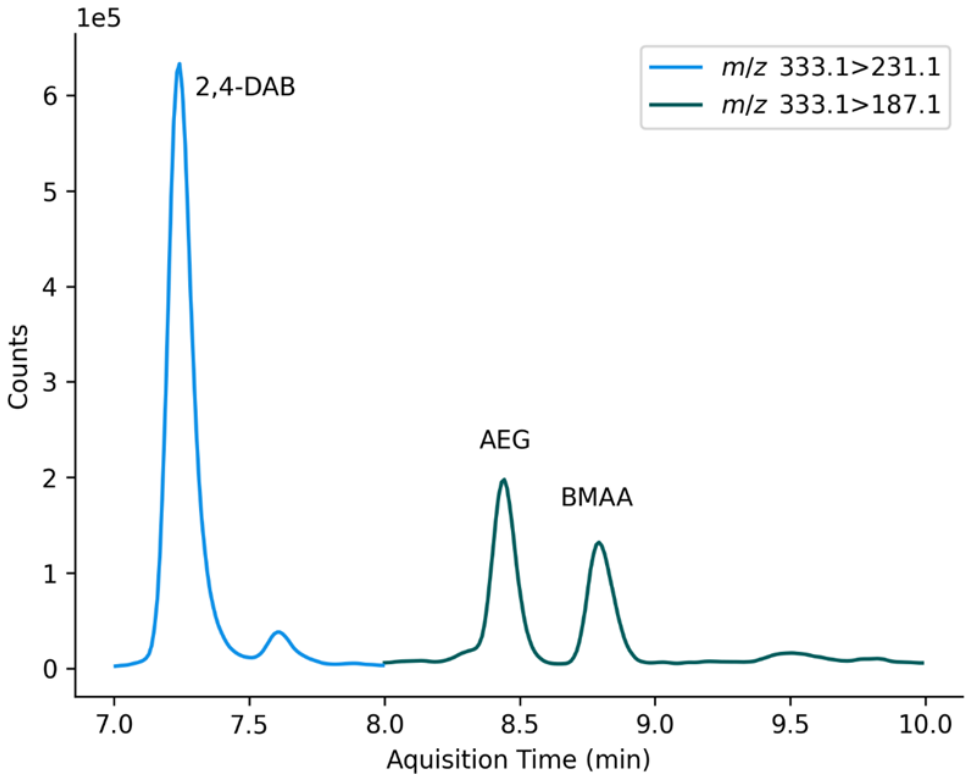
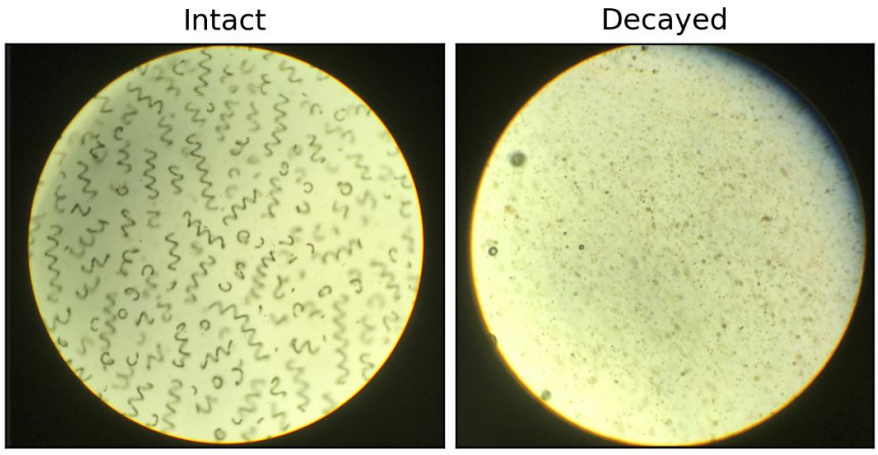



**Figure 2.3** Representative chromatogram of quantitative ion transitions for 2,4-DAB (m/z 333.1 → 231.1), AEG (m/z 333.1 → 187.1) and BMAA (m/z 333.1 → 187.1) for an insoluble protein-associated sample from 29/01/2020.

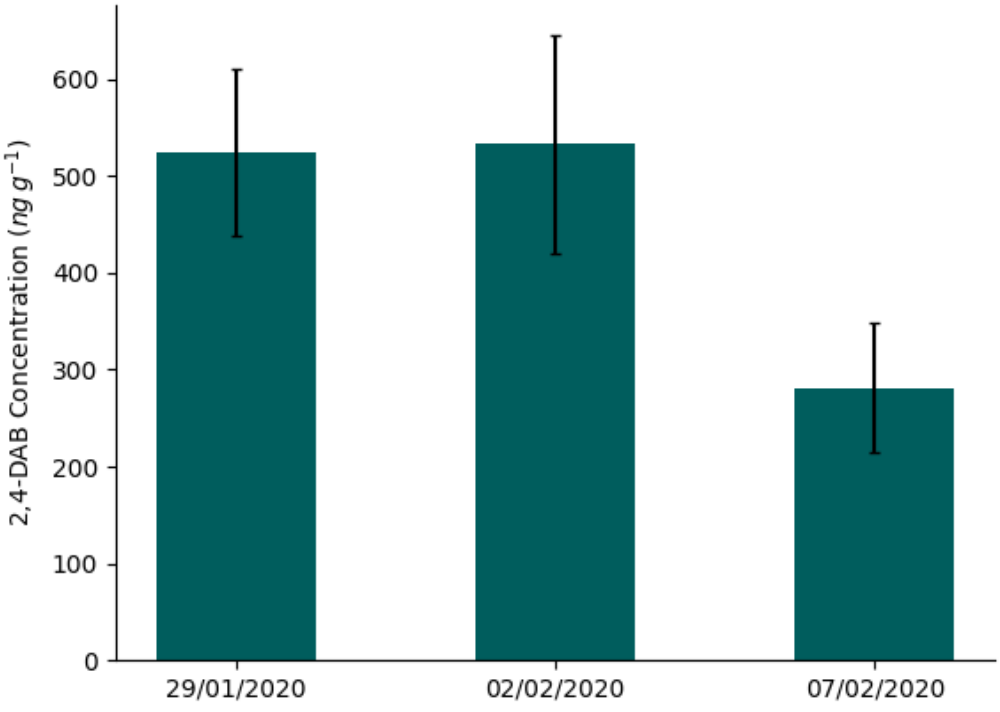
## 2.5 Conclusions

The concentrations of BMAA and its isomers changed in samples collected across three time points over the period of a developed cyanobacterial bloom to the final stages of the bloom. BMAA and 2,4-DAB decreased in concentration as the bloom collapsed, while AEG increased. The concentrations of all isomers were highest in the insoluble fraction. At the final time point where intact and lysed samples were selectively collected, BMAA did not show a difference in concentration, whereas 2,4-DAB had much higher concentrations in the lysed cells. The changing concentrations of all three isomers demonstrates the need for taking multiple samples through a bloom event, and the need for improved sample preparation and analysis to measure BMAA in water samples.

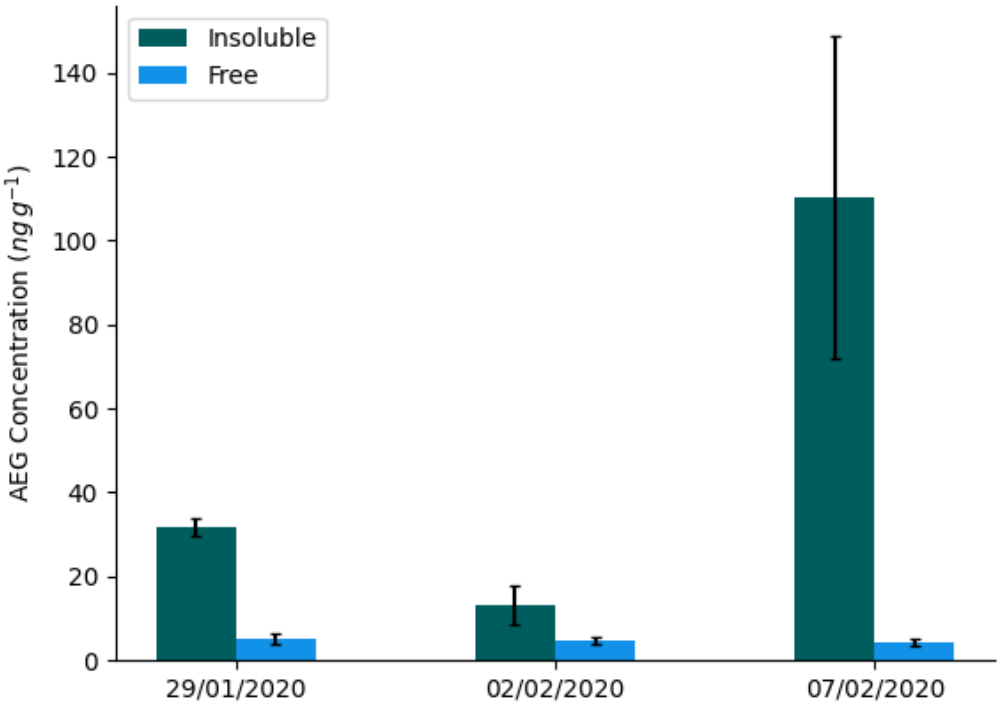
## 2.6 Supplementary information




**Supplementary Figure 2.1** Micrograph of the two samples taken from 07/02/2020 at 100x magnification. “Intact” is the sample taken from the bulk of the bloom, “Decayed” was selectively sampled from a part of the bloom with cell breakdown.




**Supplementary Figure 2.2** 2,4-DAB concentrations in the insoluble fraction ± standard deviation in environmental cyanobacteria samples (n=3, n=6 for 07/02/2020 with combined decayed and the intact results).




**Supplementary Figure 2.3** AEG concentrations ± standard deviation in environmental cyanobacteria samples (n=3, n=6 for 07/02/2020 with combined decayed and the intact results).

# Chapter 3: Method development for analysis of BMAA in mussel tissue

Accurate analysis of BMAA requires high sensitivity and selectivity to ensure reliable detections in environmental samples. The methods used previously in Chapter 2: proved unsuitable for the complex matrix of mussel tissue, necessitating tailored optimisation for this sample type. This chapter addresses the second thesis aim by detailing the development and optimisation of a BMAA analysis method specific to mussel tissue. Key components of the method, including hydrolysis protocols, sample clean-up, derivatisation agents, internal standardisation, and instrumental analysis techniques, were systematically compared and optimised to maximise sensitivity and selectivity for BMAA detection in this bioindicator species.

## 3.1 Introduction

The reliable detection and quantification of BMAA in biological matrices, such as mussel tissue, pose several analytical challenges. BMAA is often present in trace concentrations, which, when coupled with the complexity of biological matrices, requires both highly sensitive and selective methods of analysis.[116, 167] The analysis is further complicated by the existence and co-occurrence of its isomers, such as 2,4-DAB, AEG and BAMA, which can contribute to false positive detections without careful chromatographic separation.[52] Analytical protocols must also account for BMAA's presence in both free and bound forms. Bound forms are particularly problematic, as BMAA makes up only a small fraction of the total amino acid content in these fractions and is more susceptible to matrix effects.[167] This further increases the potential for interference and false negatives without sensitive and selective analytical methods. To achieve high confidence in results, a method that can successfully separate the target from interferences is required.[122] One approach to this is through the use of sample preparation.

There are currently several different approaches to analysing BMAA, including different sample clean-up procedures (no clean-up,[115] dSPE,[40] or SPE[167]), derivatisation (no derivatisation,[115] AccQ-Tag,[108] PCF,[40] DNS,[118] or FMOC[66]), and internal standards (isotopically labelled 2,4-DAB or BMAA). While these different approaches are sometimes sample specific, such as using SPE to preconcentrate water samples, methods are often used without justification behind selection or thorough validation.[82, 122] This has led to a variety of different methods being used for similar samples.

One example is the different approaches to analysing BMAA in mussel tissue. Some methods integrate steps like derivatisation[118] and SPE[167] to improve sensitivity, while others opt for a more direct analysis. [145, 168, 169] In these studies, SPE is dismissed due to the observed reduction in signal.[160, 170] However, this conclusion is made without the use of internal standards, which are needed for accounting for losses during sample preparation and without assessing matrix effects. [160, 170] Without pre-concentration, SPE is always accompanied by some signal loss, but typically increases overall sensitivity due to the improved signal-to-noise that is associated with reduced interferences.

Due to these methodological differences, evaluating the suitability of each approach for complex matrices remains challenging based on the literature alone. Unlike simpler samples, complex biological matrices, such as mussel tissue, require tailored methods to achieve both sensitivity and accuracy. This chapter outlines the optimisation of an analytical method designed to achieve high sensitivity and specificity for BMAA detection in mussel tissue. Optimisation efforts focused on assessing several key parameters, including:

- Protein hydrolysis
- Solid phase extraction
- Derivatisation
- Internal standardisation
- Analytical instrumentation

Sample preparation was optimised through protein hydrolysis, solid-phase extraction (SPE), and derivatisation, with three derivatisation agents, PCF, DNS, and AccQ-Tag, evaluated for their effectiveness in the quantification of BMAA. Instrumental analysis methods, including LC-MS/MS and GC-MS/MS, were also optimised and compared, with each technique's capacity to resolve BMAA from its isomers, minimise matrix interference, and achieve low detection limits assessed. This study establishes a robust analytical protocol suitable for bioindicator studies in complex environmental samples, advancing our capacity to monitor BMAA exposure and its associated environmental risks.

## 3.2 Experimental

### 3.2.1 Reagents and materials

*Velusinio ambiguus* mussels were purchased from Australian Aquatic Biological (Karuah, NSW, Australia). 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 Merck (Bayswater Vic, 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) and 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 Merck (Bayswater Vic, Australia). The $d_5$DAB internal standard had multiple deuteration states, and $d_3$ was found to be the most abundant. Methanol and acetonitrile were both LiChrosolv® hypergrade for LC-MS, and purchased from Merck (Bayswater Vic, 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 Merck (Bayswater Vic, Australia). HPLC grade ethyl acetate and ammonium hydroxide, acetone and dansyl chloride were also purchased from Merck (Bayswater Vic, Australia). Solid phase extraction

cartridges were Oasis MCX, 3cc 60mg, and AccQ-Tag Ultra Derivatization Kit, both from Waters (Rydalmere, NSW, Australia). The EZ: Faast™ amino acid analysis kit was purchased from Phenomenex® (Sydney, NSW, Australia).

### 3.2.2 Samples and standards

Mussel tissue was removed from the shells, and freeze-dried. Approximately 50mg of dry mussel tissue was lysed by probe sonication in 1mL 10% TCA and 20 µL of internal standard mix (500 ng/mL of d5DAB and d3BMAA). Samples were left overnight at 4°C for proteins and other insoluble biomolecules to precipitate. Samples were centrifuged (3000×*g* for 20 min), with the resulting supernatant combined with a further pellet wash of 400 µL 10% TCA. The combined supernatants (soluble fraction) were freeze dried overnight. The soluble fraction was reconstituted in 400 µL 10 mM HCl and was filtered (Ultrafree-MC LG Centrifugal 0.2 µL PTFE Membrane Filter). Meanwhile the protein pellet (insoluble fraction) was transferred to a glass vial for protein hydrolysis.

### 3.2.3 Protein hydrolysis

Glass vials containing the insoluble sample pellet were placed into hydrolysis vials along with 1 mL HCl, and the air was removed by vacuum, and replaced with nitrogen three times. Hydrolysis was then completed by either oven or microwave (Table 3.2) for optimisation. Microwave hydrolysis was completed using a Milestone ETHOS X microwave extractor (Ethos X). Oven hydrolysis at 110 °C for 16 hours was used for all other samples. Upon completion, hydrolysis vials were left briefly to cool, and the pressure was released. Samples were then removed, reconstituted in 400 µL of 10mM HCl and filtered (Ultrafree-MC LG Centrifugal 0.2 µL PTFE Membrane Filter)

### 3.2.4 Solid phase extraction

Sample clean-up was completed using Waters MCX SPE cartridges (3cc, 60 mg). Unless otherwise described during the optimisation of each step, 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.

### 3.2.5 Derivatisation

Mussels and standards were derivatised using propyl-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 and 100 µL of ethyl acetate was added, before being 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 nitrogen and were reconstituted 100 µL of 50% MeOH in water.

Derivatisation was also completed by AccQ-Tag, as described by Pravadali-Cekic et al.[102], DNS as per Salomonsson et al.[118], and EZ:Faast PCF as per Peters et al.[171] for comparisons to the optimised method. All other samples were derivatised using the above PCF method.

### 3.2.6 Instrumentation

Samples were analysed using either LC-MS/MS or GC-MS/MS.

GC-MS/MS analysis was performed on a Shimadzu GCMS-TQ8050 NX, with a Rtx-5MS 30m x 0.25mm, 0.25 µm GC column. Splitless injections were performed with an injection volume of 1µL, at 280 °C. The column flow rate was

1.4 mL/ min, and the total run time was 13 min, including a 6 min solvent delay. The column temperature was programmed to start at 90 °C and was held for 1 min, followed by an increase of 25 °C/min until reaching a final temperature of 290 °C, which was held for 4 mins. Mass spectrometry was performed in MRM mode, with transitions listed in (Table 3.1). Data analysis was performed with Shimadzu's GCMS Lab Solutions Postrun Analysis. Limits of detection were calculated using a 0.1 µg /L standard, using the signal-to-noise ratio ≥ 3.

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) and a 5 mm guard column. The column temperature was maintained 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 was maintained for the first 5 minutes, before being increased to 90% B until 7 minutes. The mobile phase then returned to the initial conditions to equilibrate before the next injection for 1.5 mins. 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. Heat block temperature of 400 °C and a drying gas flow rate of 10.00 L/min were used. Multiple reaction monitoring (MRM) mode was used with specific ion transitions and collision energies detailed in (Table 3.1). Data analysis was performed using Shimadzu's Lab Solutions.

**Table 3.1** GC-MS/MS and LC-MS/MS MRM parameters for BMAA, AEG and 2,4-DAB

| | | Precursor Ion (*m/z*) | Product ion (*m/z*) | Collision energy (eV) |
|---|---|---|---|---|
| GC-MS/MS | BMAA | 130 | 44 | 6 |
| | | | 88 | 6 |
| | | 187 | 145 | 9 |
| | AEG | 130 | 44 | 6 |
| | | | 88 | 6 |
| | | 88 | 60 | 6 |
| | 2,4-DAB | 142 | 56 | 12 |
| | | 143 | 101 | 6 |
| | | 203 | 143 | 6 |
| LC-MS/MS | BMAA | 333.2 | 187.1 | -20 |
| | | | 159.2 | -18 |
| | | | $73.2^{+}$ | -14 |
| | AEG | 333.2 | 187.1* | -16 |
| | | | 99.1 | -27 |
| | | | 88.1 | -30 |
| | 2,4-DAB | 333.2 | 231.1 | -15 |
| | | | 187.1 | -19 |
| | | | $99.1^{+}$ | -11 |

The final, optimised methods are described in full in section 4.2 Methods & Materials.

## 3.3 Results & discussion

The methods in Chapter 2: used the commercial sample preparation and derivatisation kit, EZ:Faast for the preparation of cyanobacterial samples for BMAA analysis. The EZ:Faast kit is a commercial sample preparation and derivatisation tool primarily designed for simplifying the analysing of the 20 protein amino acids.[172] Although the kit can be used for over 50 amino acids,

and therefore includes some NPAAs, BMAA and its isomers were not included among these. The EZ:Faast kit was developed for samples including urine, plasma and some foods, rather than the environmental matrices relevant to BMAA analysis. While there are many successful analyses utilising the EZ:Faast kit to detect BMAA in phytoplankton samples,[21, 26, 40, 42, 112] there is only one study that has used this method for more complex environmental samples.[112] This study compared different BMAA analysis methods, including EZ:Faast, for different matrices including cyanobacteria and blue crab tissue. While BMAA detections were reported for both matrices, no quantification was done, and no internal standard was used. [112]

To test the suitability of the EZ:Faast kit for mussel tissue, samples were spiked with 5 ng/mL BMAA, and analysed with the methods used in Chapter 2:.[171] No BMAA was observed in these spiked samples, indicating considerable matrix suppression (Figure 3.1). This suggests that the EZ:Faast dispersive SPE (dSPE) clean-up (using a proprietary cation-exchange sorbent) is insufficient for complex matrices like mussel tissue. This limitation, along with the kit's discontinuation, presented the need to develop a more robust method capable of reliably detecting BMAA in complex, environmentally relevant matrices, and in particular, mussel tissue.

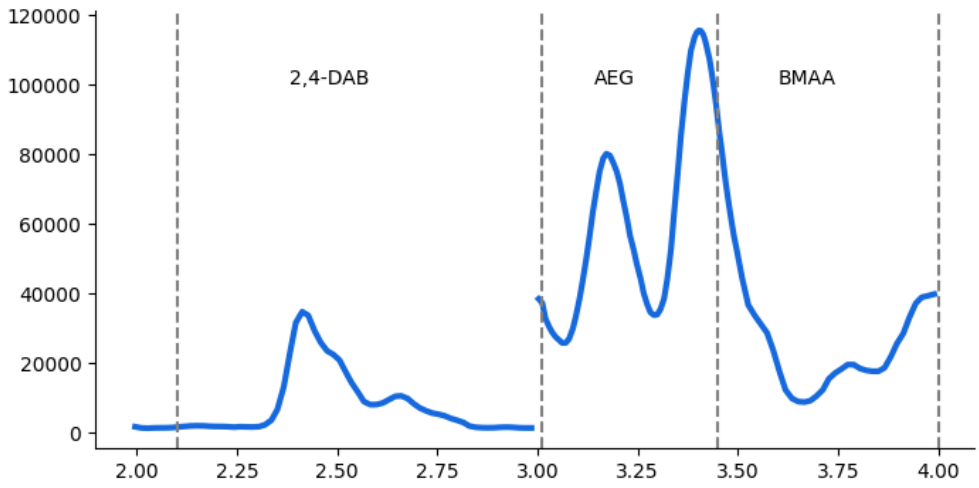



**Figure 3.1** Representative chromatogram of mussel tissue spiked with 5 ng/ mL BMAA and 2,4-DAB using EZ:Faast.

Subsequent method development, therefore, focused on optimising each sample preparation and analysis step. Specifically, protein hydrolysis, SPE, derivatisation, use of internal standards and comparing analytical instrumentation.

### 3.3.1 Protein hydrolysis

BMAA is typically either found as a free form, which is readily extracted by common solvents, or bound into larger biomolecules. To release bound BMAA, these biomolecules must be broken down into their constituent amino acids. The accepted standard procedure for releasing bound BMAA involves a 16-hour oven hydrolysis process with 6 M HCl in either liquid or vapour phase.[115, 160, 173] The 16-hour oven hydrolysis is based on methods used to release amino acids from proteins. However, how BMAA is associated with larger biomolecules is not known and could be dependent on the sample matrix. Some studies show BMAA associated with large molecular weight compounds such as proteins,[174, 175] while others suggest presence in smaller molecules such as peptides.[168, 176] Therefore, further optimisation of hydrolysis could be beneficial to increasing recoveries and improving the accuracy of BMAA extraction. Microwave-assisted hydrolysis offers a promising alternative to oven hydrolysis by potentially reducing hydrolysis time from an overnight process to as little as 5 minutes,[177] presenting an opportunity to significantly reduce the sample preparation time, whilst optimising parameters to efficiently hydrolyse bound BMAA.

Previous investigations into optimising microwave-assisted hydrolysis for BMAA extraction across two sample matrices found increased BMAA recovery in cyanobacterial samples and SH-SY5Y cell samples when compared to the traditional oven hydrolysis.[178] However, the optimal hydrolysis conditions were unique to each sample matrix, indicating that matrix composition impacts the most effective extraction parameters and, therefore, likely the way BMAA is bound into biomolecules.

Unfortunately, there are difficulties with optimising and validating this process, as standards, being freely soluble rather than bound, cannot be used. Using samples for method development also has its limitations, as samples known to have bound BMAA must be used. Many samples don't have detectable bound BMAA, which could be a result of inefficient hydrolysis or suppression from the complex matrix rather than no bound BMAA being present.

Traditional oven hydrolysis was compared to three different microwave hydrolysis methods, with the aim of accurately quantifying BMAA in the insoluble (bound) fraction. Triplicate samples were run for each different hydrolysis method. Four hydrolysis methods were run (Table 3.2), each with n=3 mussels. However, no bound BMAA was detected in any of the mussels analysed, with any hydrolysis method. This is possibly a limitation of having a positive control to use for method development and would benefit from further exploration if an appropriate positive sample is identified. As no BMAA was detected in the hydrolysed fraction, the traditional oven hydrolysis method was selected for future works, as microwave hydrolysis could not be validated.

**Table 3.2** Oven and microwave hydrolysis parameters

| | Temperature (°C) | Time |
|---|---|---|
| Oven | 110 | 16 hours |
| Microwave | 100 | 45 min +10 min ramp |
| Microwave | 110 | 45 min +10 min ramp |
| Microwave | 120 | 45 min +10 min ramp |

### 3.3.2 Solid phase extraction

Substantial sample clean-up is required to limit the impacts of matrix effects when trace concentrations of BMAA are present in complex sample matrices. The previously used EZ:Faast kit included a (dSPE) step. However, this was not sufficient clean-up for the mussel tissue samples, as BMAA was unable to be

detected in spiked samples. SPE using Waters Oasis MCX mixed-mode cation cartridges was selected, as it enables effective sample clean-up by combining cation exchange and reverse-phase mechanisms. It has also previously been shown to be a useful tool for BMAA extraction from multiple different sample matrices.[64, 167, 179]

SPE protocols were optimised by assessing the load, wash and elution conditions with mussel tissue spiked with 5 ng/mL BMAA, 2,4-DAB and AEG. Given BMAA's zwitterionic nature at neutral pH, an acidic pH near the lowest pKa was chosen for the starting load conditions to ensure retention with the cation exchange sorbent (Figure 3.2). HCl concentrations at 2, 10, 50 and 100 mM were tested to optimise retention (Figure 3.3). For elution, various

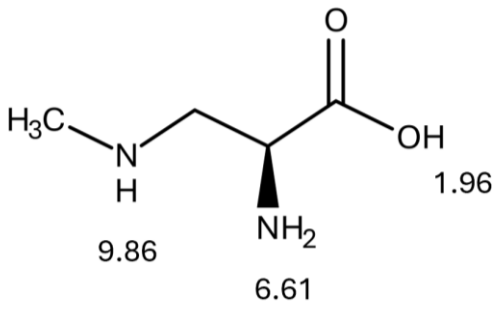



**Figure 3.2** pKa values of BMAA

concentrations of $NH_4OH$ in MeOH (5%, 7%, 10% and 13%) were tested to find the most efficient elution strength necessary for BMAA recovery while minimising variability. An optimal elution concentration was selected by examining both peak area and variance (Figure 3.3).

Loading concentrations of 10mM HCl, and elution in 10% $NH_4OH$ in MeOH were selected for providing both the highest signal (peak area) and for having the most reproducible results. These values are also beneficial for ensuring robustness, as there is no significant change in response between the selected concentrations and those assessed on either side, ensuring any variations in solvent concentration have minimal impact on the final results.

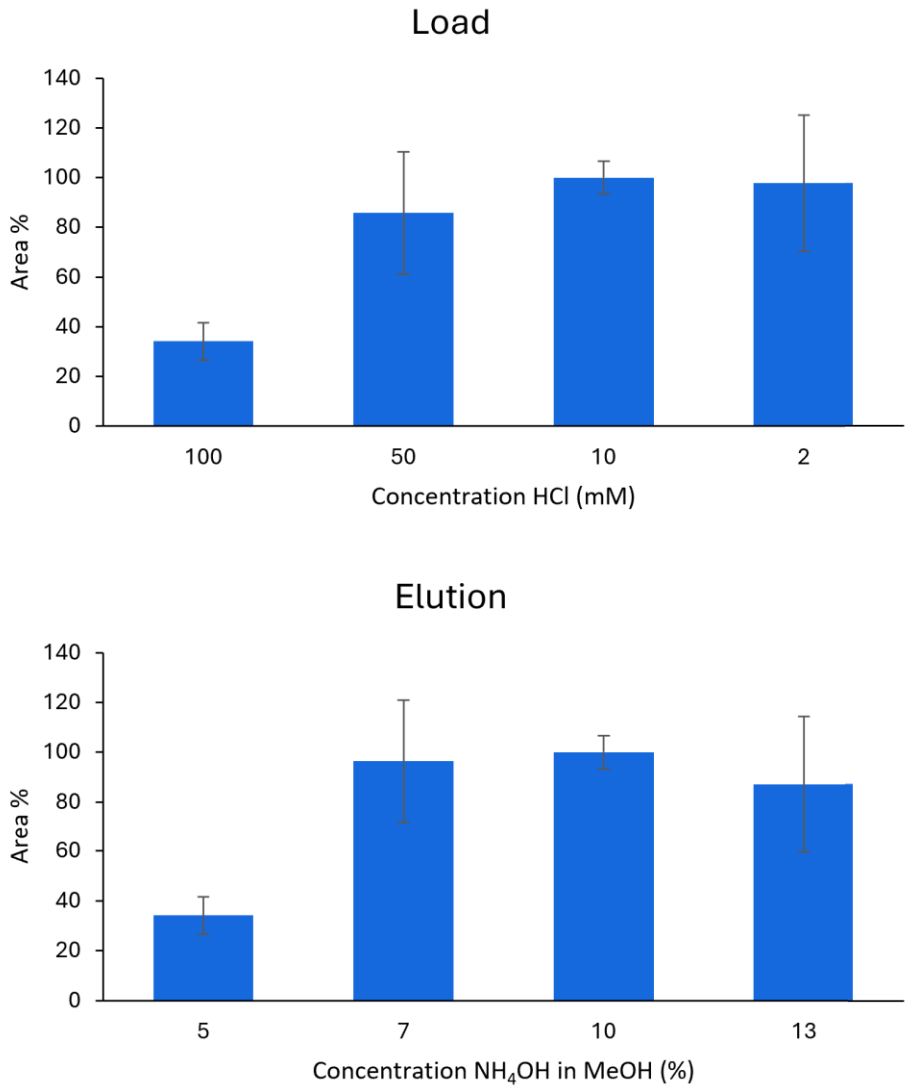



**Figure 3.3** Results of different SPE conditions for loading (top) and eluting (bottom). Areas are shown as a percentage of the final selected condition.

Due to the particularly complex matrix of the insoluble (bound) fraction, an additional wash was required prior to elution for this fraction to reduce interferences. A wash of 2% $NH_4OH$ in MeOH suitably increased the S:N ratio, improving limits of detection by 7x (Table 3.3). However, this wash was not needed for the soluble (free) fraction, and so it was not used for that fraction.

**Table 3.3** Improvements in signal to noise ratio (S/N) with increasing concentrations of $NH_4OH$ in additional wash step for the bound fraction.

| % $NH_4OH$ | BMAA S/N |
|---|---|
| No wash | 19.75 |
| 0.5% | 49.93 |
| 1% | 61.18 |
| 2% | 133.66 |

Sample loading volume and concentration were optimized to ensure effective retention without overloading the cartridge. Mussel tissue concentrations of 12.5, 25, and 37.5 mg dry weight (DW)/ mL were tested, with 25 mg/mL proving most effective. This loading concentration aligns with previous findings in amino acid extraction studies, which recommend a 1:50 extraction ratio (20 mg dry weight mussel tissue per mL) for optimal retention efficiency and clean-up.[167] Final loading was achieved with 200 µL sample volume in 1 mL loading solution.

### 3.3.3 Derivatisation

Derivatisation of BMAA enables analysis by RPLC by reducing the polarity, and improving MS sensitivity by increasing its molecular weight. There are several different approaches to BMAA derivatisation, including commercial and in-house protocols, and utilising ethyl-chloroformate,[180] PCF,[21, 26, 42] AQC,[59, 102, 108] DNS,[118, 120, 181] or FMOC[66] as derivatising agents. To optimise the derivatisation of BMAA, three methods were compared to the EZ:Faast kit. The derivatisation agents that were assessed for suitability were PCF, AccQ-Tag and DNS.

#### 3.3.3.1 AccQ-Tag

AccQ-tag is a commercially available kit, that utilises derivatisation by 6-aminoquinoly-N-hydroxysuccinimidyl carbamate (AQC). It is the most popular approach for analysing derivatised BMAA, and it has been used for a large

variety of sample matrices. Therefore, the suitability of using this method for mussel analysis was assessed.

Free and hydrolysed samples were spiked to three different levels (1, 5, and 25 ng /mL), underwent the optimised SPE protocol, and were analysed using the LC-MS/MS method described in Pravadali-Cekic et al.[102] with some slight modifications. Calibration standards ranging from 0.5-50 ng/mL were also run, and were used to establish the method detection limits (Table 3.4). While the method detection limit of 0.375 ng/mL was considered acceptable for BMAA analysis, no BMAA was detected in any of the spiked mussel tissue. This indicates a limit of detection greater than 25 ng/mL in mussel matrix, which is at least 25 times higher than those achieved with PCF derivatisation. Additionally, AccQ-tag does not facilitate unique MRM fragmentation for the isomers BMAA, AEG and 2,4-DAB. Derivatisation with AccQ-tag was not considered the most suitable method due to insufficient selectivity and sensitivity and was therefore not explored further.

#### 3.3.3.2 Dansyl chloride

Dansyl chloride derivatisation was completed based on the previously reported method by Salomonsson et al.[118] where DNS was used to analyse BMAA in mussel tissue. This method was initially developed by Salomonsson et al.[118] in response to concerns that the discrepancies in BMAA concentrations reported could linked to the widely used AccQ-Tag method.

This study found concentrations as low as 0.27 ug BMAA /g WW mussel and had complete method validation and use of $d_3$BMAA as an internal standard.

A 100 ng/mL standard was derivatised as described in Salomonsson et al.[118] in duplicate. One of the standards was dried upon completion under a gentle stream of nitrogen and reconstituted in ACN to more closely match the solvents used during LC-MS/MS analysis. 2 µL Direct injections were run with a mobile phase of 80% ACN, 20% $H_2O$ both with 0.1% formic acid. The MS was run in scan and product ion scan (Q3 scan) mode. However, the 585 *m/z* that is reported in other studies was not detected in either standard. It is suspected that

derivatisation with DNS did not occur, as there was no evidence of BMAA derivatised with DNS being present in the injected standard.

**Table 3.4** Limits of detection of BMAA and 2,4-DAB with different derivatisation techniques

| | | PCF | AccQ-Tag | DNS | EZ:Faast |
|---|---|---|---|---|---|
| BMAA | Method LOD (ng/ml) | 0.025 | 0.375 | ND | 0.1 |
| | Sample LOD soluble (ng/ml) | 0.05 | >25 | ND | >25 |
| | Sample LOD insoluble (ng/ml) | 1 | > 25 | ND | >25 |
| 2,4-DAB | Method LOD (ng/ml) | 0.1 | 0.6 | ND | 0.5 |
| | Sample LOD soluble (ng/ml) | 0.5 | >25 | ND | >25 |
| | Sample LOD insoluble (ng/ml) | 1 | > 25 | ND | >25 |

#### 3.3.3.3 Propyl-chloroformate

The derivatisation of amino acids using alkyl-chloroformates is a common approach and it is the derivatisation mechanism utilised by the EZ:Faast kit applied in Chapter 2: Derivatisation requires an alkyl-chloroformate and alcohol, as well as either 3-methylpyridine or pyridine as a catalyst (Figure 3.4). [182] The alcohol must be selected to match the alkyl group on the chloroformate, to prevent two different derivatives from forming. [183]

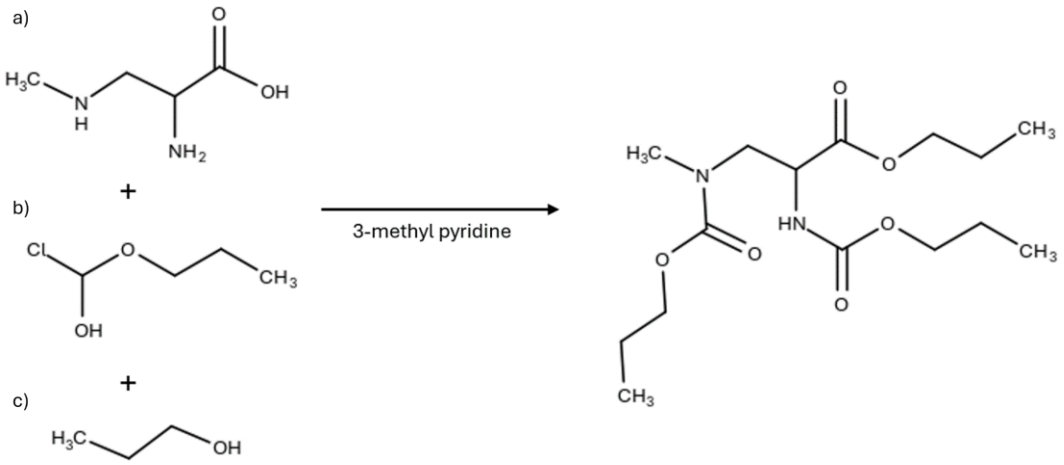



**Figure 3.4** Derivatisation reaction for BMAA (a) with propyl-chloroformate (b) and n-propanol (c)

The highest derivatisation efficiencies when comparing the different alkyl-chloroformates is typically found with butyl- or propyl-chloroformate.[182, 184] PCF was selected for optimisation due to its availability, and for ease of utilisation and comparison to the EZ:Faast kit. PCF derivatisation was based on the EZ:Faast method,[21, 26, 171] and previous studies derivatising amino acids with alkyl-chloroformates. [182-184]

One step that was altered was the pH of the sample prior to the reaction. Other studies have samples present in a sodium hydroxide solution as the derivatisation reaction produces HCl. However, the samples in this workflow are dried after SPE, and must be reconstituted prior to derivatisation. As BMAA and its isomers are poorly soluble in basic conditions, the reconstitution must be acidic. The pH of the sample solution was assessed, by adding NaOH to the sample after reconstitution in 10 mM HCl. No differences in derivatisation efficiency were observed at different pH. Therefore, reconstitution was done in 100 µL 10 mM HCl, and no further buffering was done prior to derivatisation.

The final PCF derivatisation method had a limit of detection of 0.025 ng/ mL (Table 3.4), which is 4x more sensitive than the EZ:Faast kit. While it is a relatively similar derivatisation protocol, it includes slightly more catalyst mix per sample (100 µL vs 80 µL of 20% 3-methylpyridine in 1-propanol), and is

extracted with 2x 100 µL of ethyl acetate, rather than the 100 µL of 90% iso-octane and 10% chloroform that is used in the final liquid extraction of EZ:Faast, both of which could contribute to the increased sensitivity. This derivatisation method was also the only one able to detect BMAA in spiked mussel tissue. Therefore, all further analyses used this PCF derivatisation.

### 2.2.5 Internal standardisation

Accurate quantification of BMAA in biological matrices requires a reliable internal standard (IS) to correct for any matrix effects present and account for variability and loss in the extensive sample preparation procedures that are needed for sufficient sample clean-up. In LC-MS/MS (and other MS analyses) matrix effects can significantly impact analyte response, primarily as a result of ion suppression, where MS ionisation is impacted by co-eluting compounds. This effect is often unpredictable in biological and environmental matrices, making it challenging to achieve accurate quantification.[185] To reduce matrix effects, sample preparation is often included, to clean-up the sample prior to analysis. Isotopically labelled IS have become an essential tool to account for matrix effects and any losses that occur during sample preparation procedures. As isotopically-labelled IS co-elute with the analyte and are, therefore, subject to identical suppression or enhancement, they allow for more reliable quantification by normalising the analyte response to the IS response, regardless of sample matrices.[186]

Previously, $d_5$DAB was commonly used as an IS for BMAA analysis due to commercial availability,[21, 56, 70] while studies that used $d_3$BMAA required in-house synthesis.[82, 187, 188] While studies using $d_5$DAB as an internal standard in cyanobacterial samples had acceptable recoveries, differences in matrices between cyanobacteria and mussel tissue may impact accuracy. As the newly commercially available $D_3$BMAA would co-elute with BMAA, any potential differences in matrix effects should be minimised by using this IS. The differences when normalising to $d_3$BMAA and $d_5$DAB show the remaining impacts of matrix effects and suggest differences in ion suppression between

the two retention times (Table 3.5). Therefore, selecting a suitable internal standard is necessary, and the need can vary between matrices. It is also important to consider internal standards when analysing new sample types. While $d_3$BMAA is more suitable for BMAA analysis, it is similarly a poor IS for 2,4-DAB concentrations and $d_5$DAB should also be used if quantification of 2,4-DAB is required.

**Table 3.5** Accuracy of BMAA, 2,4-DAB and AEG when normalised to the respective internal standards ($d_3$BMAA and $d_5$DAB). Calculations were done using a 5 ng /mL spike of BMAA and 2,4-DAB in mussel tissue.

| | % Accuracy $D_3$BMAA | % Accucay $D_5$DAB |
|---|---|---|
| BMAA | **105±5%** | 69±21% |
| 2,4-DAB | 59±30% | **103±2%** |

## 2.2.6 Instrumental analysis

### 2.2.6.2 LC-MS/MS

LC-MS/MS is widely accepted as the golden standard for BMAA analysis. While BMAA has been analysed by other techniques, LC-MS/MS offers distinct advantages in differentiating isomers and providing high levels of sensitivity. The LC-MS/MS method that was used for analysing BMAA derivatised by PCF was based on the methods previously used with EZ:Faast sample preparation.[171]

RPLC-MS/MS was selected as the analysis method for mussel tissue derivatised with PCF as it enabled the high levels of sensitivity and selectivity that are necessary for BMAA analysis. Future work should include comparisons to HILIC methods. HILIC is widely used, however is often used without an internal standard, and without assessing % recovery when analysing complex matrices. Additionally, an attempt at validating an underivatised HILIC method found that it failed many criteria, and that it was not fit for purpose.[119] Therefore, there is little confidence in the accuracy of these studies. Any future comparisons assessing the suitability of HILIC should still incorporate SPE clean-up prior to

analysis, and use appropriate internal standards to ensure matrix effects are accounted for.

#### 2.2.6.2 GC-MS/MS

Previous studies have used derivatisation by PCF, ethyl-chloroformate (ECF) or trimethylsilylation (TMS) to analyse BMAA by GC-MS[111, 180, 189] and two-dimensional GC-time-of-flight MS (GCxGC-TOFMS).[190] However, no published studies have yet used GC-tandem mass spectrometry (GC-MS/MS) to analyse BMAA.

Here, a selective GC-MS/MS method was developed that successfully separated BMAA from its isomers (Figure 3.5), with unique MRM transitions. GC-MS/MS method development was done with 0.1, 1, or 10 µg /mL standards containing a mix of BMAA, AEG and 2,4-DAB, which had been derivatised with the PCF method described above, with the modification of injecting the ethyl-acetate extractions without the final drying step.

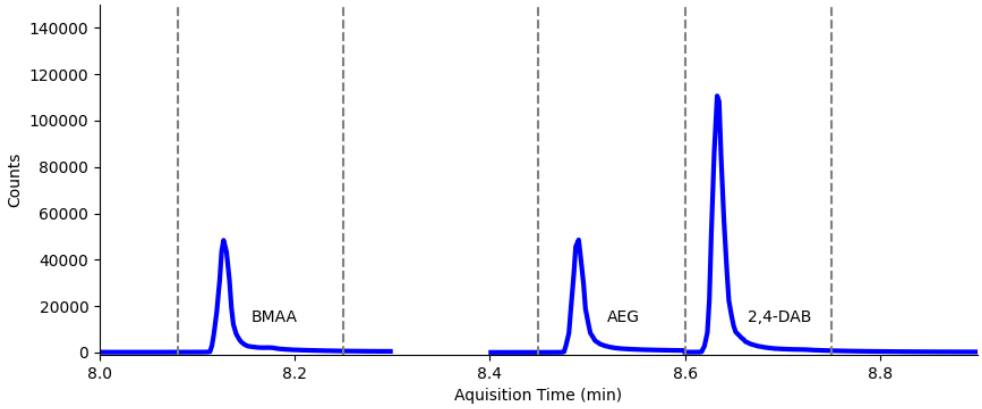



**Figure 3.5** GC-MS/MS Chromatogram (TIC) of a 1 µg /mL standard of BMAA, AEG and 2,4-DAB

While this method had acceptable selectivity, the method detection limit was determined to be 2.7 ng/mL, which is over 100 times higher than the LC-MS/MS method developed in parallel using the same derivatization protocol (Table 3.6). These higher LODs may restrict the utility of GC-MS/MS for BMAA detection when sample concentrations are expected to be in the sub-ppb range or when in complex matrices where additional matrix effects could further impact sensitivity.

**Table 3.6** Comparison of limits of detections for BMAA, AEG and 2,4-DAB by LC-MS/MS and GC-MS/MS

| Analyte | LC-MS/MS LOD (ng /mL) | GC-MS/MS LOD (ng /mL) |
|---|---|---|
| BMAA | 0.025 | 2.7 |
| AEG | 0.1 | 1.7 |
| 2,4-DAB | 0.1 | 1.8 |

### 3.2.7 Method validation

The final method followed the standard sample preparation methods previously used for cell lysis and protein hydrolysis. SPE was performed using Oasis MCX cartridges, followed by derivatisation with PCF and analysis by RPLC-MS/MS. The optimised method was validated by assessing the matrix effects, recoveries, accuracy and repeatability. Full method validation parameters are listed in Chapter 4:, Table 4.2.

The developed method is highly sensitive and accurate for BMAA analysis in mussel tissue, with in-matrix detection limits of 0.05 ng/mL for free BMAA, equivalent to approximately 0.3 ng/ g DW mussel tissue. This offers significant improvements over other recent methods analysing similar matrices, which typically report limits in the µg/g range. It also enabled the detection of BMAA in spiked mussel samples that were not able to be detected using the previously used EZ:Faast method (Figure 3.6). This method has been utilised for the following chapters when analysing BMAA in mussel tissue and has been published in Environmental Pollution, as described in Chapter 4:.[191] While matrix effects are still present, particularly in the hydrolysed insoluble fraction, normalising to the appropriate internal standard effectively accounts for this, as well as any losses throughout the sample preparation process, and provides highly accurate results.

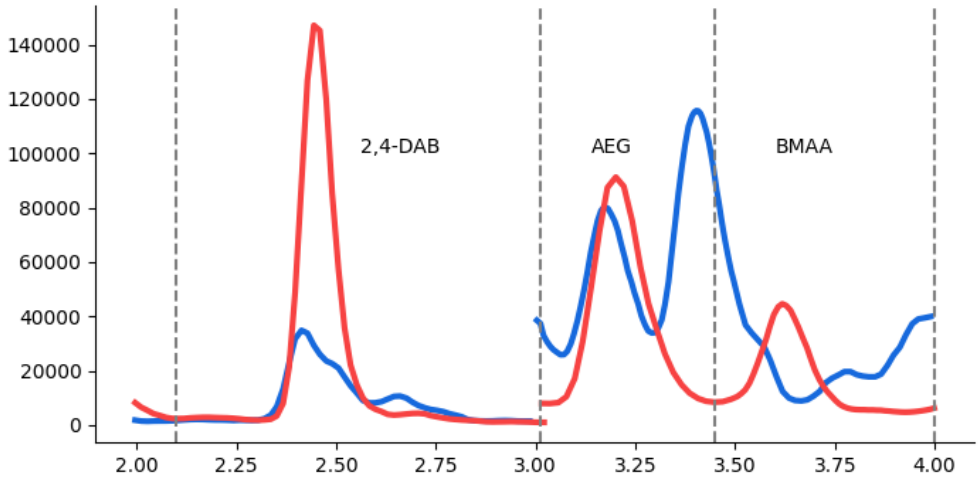



**Figure 3.6** Representative chromatograms of mussel tissue spiked with 5 ng/ mL BMAA and 2,4-DAB using newly developed method with SPE and PCF derivatisation (red) and EZ:Faast (blue).

## 2.4 Conclusions

A method was developed to accurately and confidently detect and quantify BMAA and 2,4-DAB in mussel tissue. Protein hydrolysis, SPE, derivatisation and instrumentation were all optimised. The resulting method utilised mixed-mode cation exchange SPE, derivatisation with propyl-chloroformate, and analysis by RPLC-MS/MS to achieve detection limits of 0.3 ng BMAA/g DW mussel. The method is also highly selective and can confidently differentiate between isomers. This method is fit for analysing trace concentrations of BMAA in complex sample matrices and is used for all BMAA analyses in mussel tissue in the following chapters.

# Chapter 4: Laboratory Assessment of BMAA Bioaccumulation in Mussels

## Chapter overview

In this chapter, the previously developed analytical method was applied to assessing the uptake and depuration patterns of BMAA in Australian freshwater mussels (*Velesunio ambiguus)* exposed to BMAA producing cyanobacteria (*Microcystis aeruginosa*). The goal of this work was to establish the suitability of *V. ambiguus* as bioindicators of BMAA under controlled experimental conditions. The results demonstrate that mussels accumulate BMAA in direct relation to exposure time, with concentrations rising over the exposure period and declining rapidly once exposure ended. This positive correlation suggests that mussels could be a valuable tool for biomonitoring BMAA, potentially reflecting fluctuating levels in bloom-prone environments. By confirming BMAA uptake in a controlled setting, this study provides a strong foundation for evaluating mussels as bioindicators in the field, as explored in Chapter 5:.

## Certificate of authorship

The following chapter is published in *Environmental Pollution*. I, Siobhan Peters, certify that the work in the following chapter has not been submitted as part of any other documents required for a degree.

Authorship contributions

| Author | Contributions | Signature |
|---|---|---|
| Siobhan J. Peters | methodology, validation, formal analysis, investigation, conceptualisation, writing-original draft, visualisation. | Production Note: Signature removed prior to publication. |
| Simon M. Mitrovic | conceptualisation, writing- review & editing, funding acquisition. | Production Note: Signature removed prior to publication. |
| Kenneth J. Rodgers | conceptualisation, writing- review & editing, funding acquisition. | Production Note: Signature removed prior to publication. |
| David P. Bishop | conceptualisation, writing- review & editing, supervision, funding acquisition | Production Note: Signature removed prior to publication. |

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

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Keywords:

Cyanotoxins, bioaccumulation, BMAA, bioindicators, freshwater mussels

## Highlights:

- *V. ambiguus* mussels have potential as bioindicators of BMAA.
- Mussels showed BMAA uptake when exposed to *Microcystis aeruginosa*
- Rapid depuration following exposure demands careful sampling plans *in situ*
- A sensitive method was developed to detect BMAA in mussels at 0.4 ng/g

## 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.

## 4.1 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.[192] 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.[7] 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.[13] 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.[20, 22, 25] BMAA is linked to high incidences of global neurodegenerative disease.[89] Although the acute neurotoxic effects of BMAA have been extensively observed both in vivo and in vitro,[32, 35, 90, 193] the exact mechanism contributing to neurodegeneration remains unclear. Chronic exposure to BMAA is believed to contribute to neurodegeneration,[89] 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.[37, 105, 107] AEG's toxicity remains contentious, with conflicting reports in the literature.[37, 107] 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. [25, 29, 62, 174, 194-196] Exposure through food sources is exacerbated by BMAA's ability to bioaccumulate, creating higher concentrations in food sources such as seafood.[27, 29, 175, 197]

Determining levels of BMAA exposure can be challenging. This results from a combination of difficulties with analytical methods, low concentrations, and complex sample matrices.[82] Most studies analysing BMAA in the environment focus on cyanobacterial samples.[64, 65, 82] 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 [38, 39], while others report its presence in multiple samples [40-43]. 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,[198-200] and concentrations of BMAA in cyanobacteria can quickly change across a bloom event.[171] 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.[66, 67] In other studies, the reported limits of detection for BMAA in water range between 10 and 5000 ng/L,[64, 110, 121, 201] meaning these other methods would not have been sensitive enough to be able to detect BMAA in the majority of positive water samples.[64, 66, 67, 110, 121, 201] 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.[129, 134, 202-205] They make particularly effective bioindicators due to their filter-feeding behaviour, sedentary lifestyle, long lifespan, wide distribution and bioaccumulation potential.[204] 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.[29, 30, 54, 197] 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.[28, 29, 54] BMAA concentrations in mussels has been a focus of some recent studies, due to concerns regarding direct human consumption,[197] bioaccumulation potential into livestock through use in feed, [68] and as potentially useful bioindicators for BMAA levels in aquatic environments.[145, 146, 169, 206] 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,[145, 146, 206] 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.[169] 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[207] and in the environment suggests this approach may be useful.[208-211]

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.

## 4.2 Methods & Materials

### 4.2.1 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) and 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 $d_5$DAB internal standard had multiple deuteration states, and $d_3$ 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, 3cc 60mg, from Waters (Rydalmere, NSW, Australia)

### 4.2.2 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 $3.5x10^6$ 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.

### 4.2.3 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 $2x10^7$ cells/mussel/day of *Microcystis aeruginosa* MASH01-AO5 at a frequency of twice a week (Figure 4.1). This cyanobacterium

was chosen for the exposure experiment as it had previously been found to produce BMAA,[26, 40, 78] 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.

### 4.2.4 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, and 112 during the depuration period (Figure 4.1). 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.

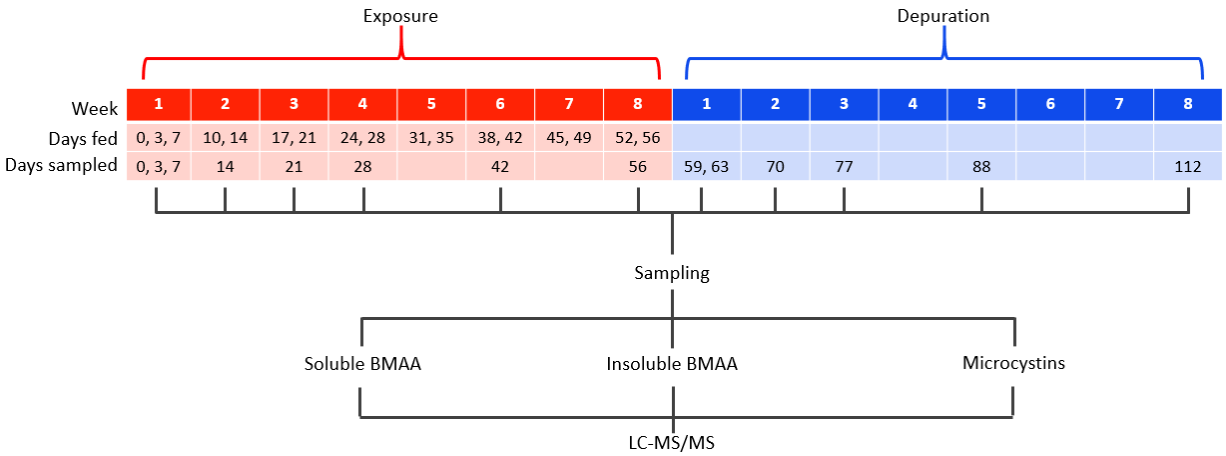



**Figure 4.1** Feeding and sampling timeline and experimental flow chart for the exposure and depuration periods.

### 4.2.5 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 $d_5$DAB and $d_3$BMAA. 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 four hours to enable protein precipitation before being centrifuged at 3000 x*g* for 20 minutes. 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 20mM HCl and transferred to 0.2 µm membrane spin filter tubes (Ultrafree-MC LG Centrifugal 0.2 µL PTFE Membrane Filter).

### 4.2.6 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 hours to undergo hydrolysis by HCl vapour. After 16 hours, 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 20mM 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 8000x *g* for 1 hour. Extracts were either stored at -20 °C or proceeded directly to the next stage.

### 4.2.7 Extraction

Sample clean-up was completed using Waters MCX SPE cartridges (3cc, 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.

### 4.2.8 Derivatisation

All samples and standards were derivatised using propyl-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.

### 4.2.9 Analysis by LC-MS/MS

Standards were made ranging from 0.025-1000 ng/mL of both BMAA and DAB, and with a final concentration of 25 ng/mL of d3BMAA and d5DAB. 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 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 minutes, after which time all target analytes had eluted and the mobile phase was increased to 90% B until 7 minutes to wash the column. The mobile phase then returned to the initial conditions to equilibrate before the next injection for 1.5 mins. 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. 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 4.1. 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.

### 4.2.10 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 minutes. Samples then underwent probe sonication at 50% intensity for 1 minute, 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.

**Table 4.1** 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* | 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* | 336.2 | 190.1 | -20 |
| | | 162.1 | -18 |
| | | $76.1^{+}$ | -14 |

* Denotes internal standards. + denotes the ion transition used for quantification.

Analysis took place using modified method from Pravadali-Cekic et al. [102] 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 × 100mm, 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 minutes. The mobile phase was held for 0.5 minutes at 32.5% B before increasing to 90% B for 1.5 minutes. The system then returned to starting conditions to equilibrate for 2 minutes before the next injection. 5 µL was injected of each sample in duplicate.

### 4.2.11 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 differences in data sets.

## 4.3 Results & discussion:

A highly sensitive and accurate sample preparation method for the analysis of BMAA was developed (Table 4.2) 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[145, 146] 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

**Table 4.2** 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** | **2,4-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% |

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 Figure 4.2, 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 the matrix is further exemplified by spiked recoveries where only peak areas were compared, showing percentage recoveries from 22-38% (Table 4.2). 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 ($r^2 = 0.77$, Figure 4.3). 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 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 that exposure could have occurred

prior to obtaining the mussels. This is consistent with some other mussel accumulation studies which assessed the uptake of free BMAA dissolved in the water, where they also found detectable. levels of BMAA in the control mussels.[145, 146] However, these studies did not observe the rapid and non-linear

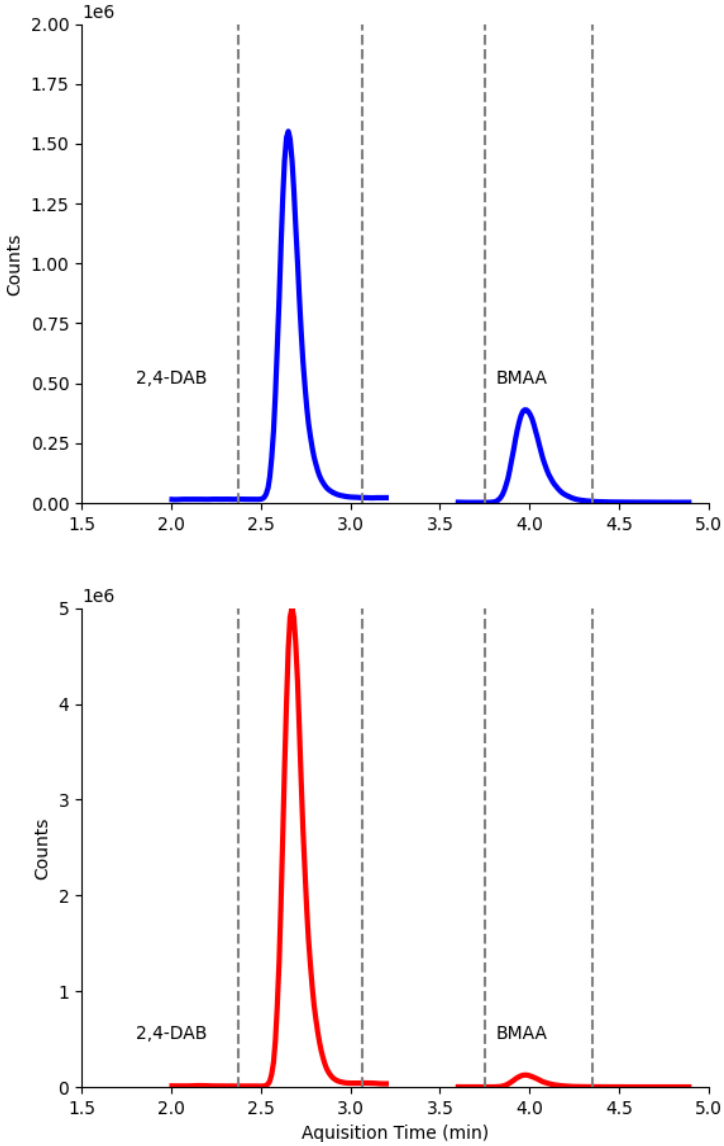



**Figure 4.2** Representative chromatograms of 2,4-DAB and BMAA using the respecti quantification MRM for each analyte. The blue line (above) is a chromatogram of a standa containing 5 ng/mL of 2,4-DAB and BMAA. The red line (below) is of a representative solut fraction of a mussel sample spiked with 5 ng/mL of 2,4-DAB and BMAA.

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.[145] 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.[145, 146] 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

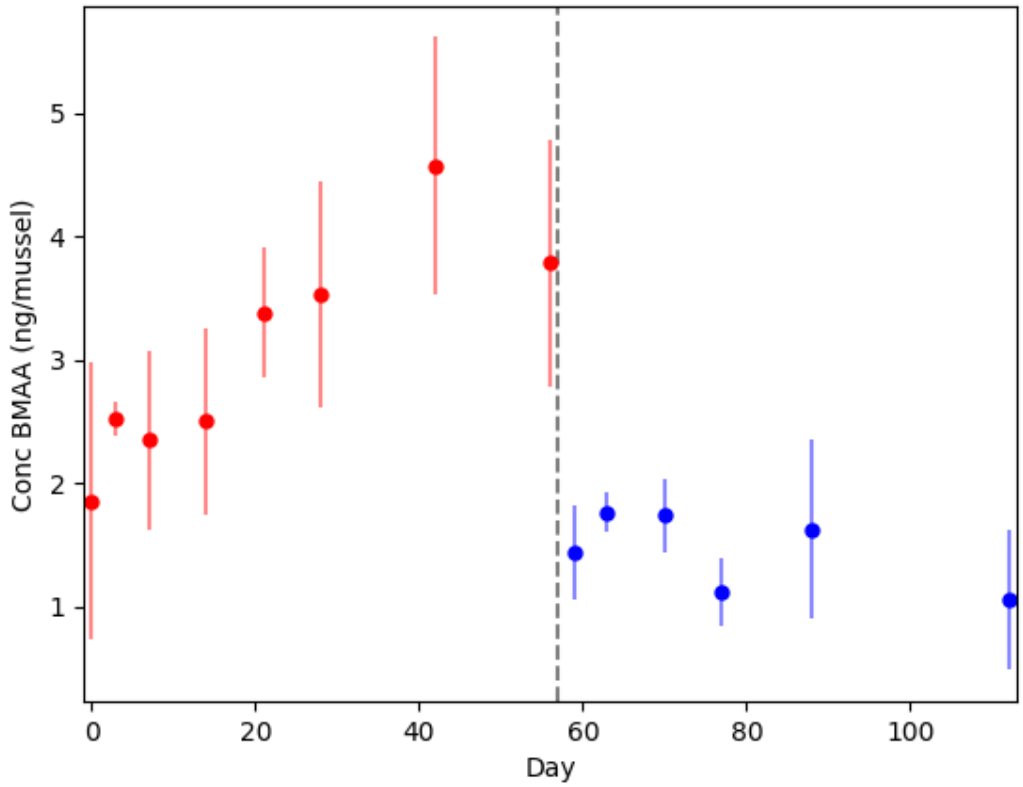



**Figure 4.3** Concentration of BMAA in mussels over the duration of the experiment. Dots denote the mean concentration and error bars are standard error. Red is the exposure period and blue is the depuration period, with the grey dashed line showing the end of the exposure period.

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.[146]

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 (Supplementary Figure 4.1) concentrations. 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 (Figure 4.4). 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.[209] This was suggested to be a result of mechanisms that enabled the mussels to reduce exposure to cyanotoxins. 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.[212]

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.[213, 214]

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[207, 209, 210, 215] 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

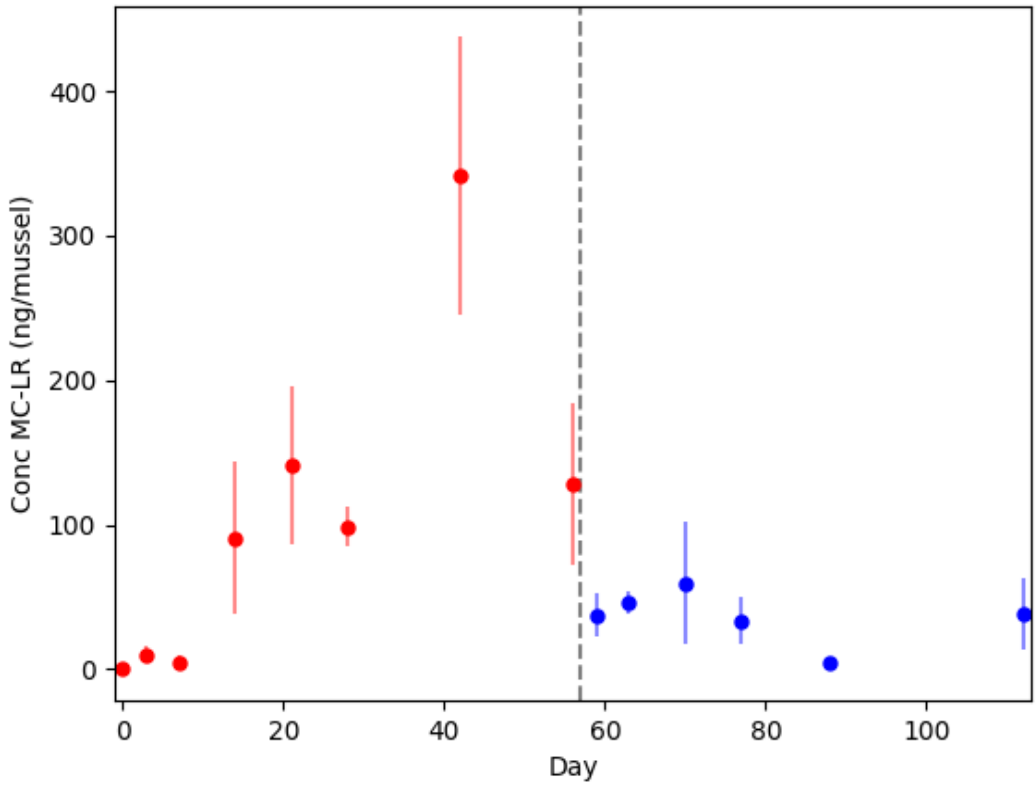



**Figure 4.4** Concentration of MC-LR in mussels over the duration of the experiment. Dots denote the mean concentration and error bars are standard error. Red is the exposure period and blue is the depuration period, with the grey dashed line showing the end of the exposure period.

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 an early 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.

The presence of 2,4-DAB in both soluble and insoluble fractions, with concentrations much higher than BMAA (Supplementary Table 4.1), 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 to prevent misidentification of BMAA (Supplementary Figure 4.2). 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.[216] 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.[217] 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.

## 4.4 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.

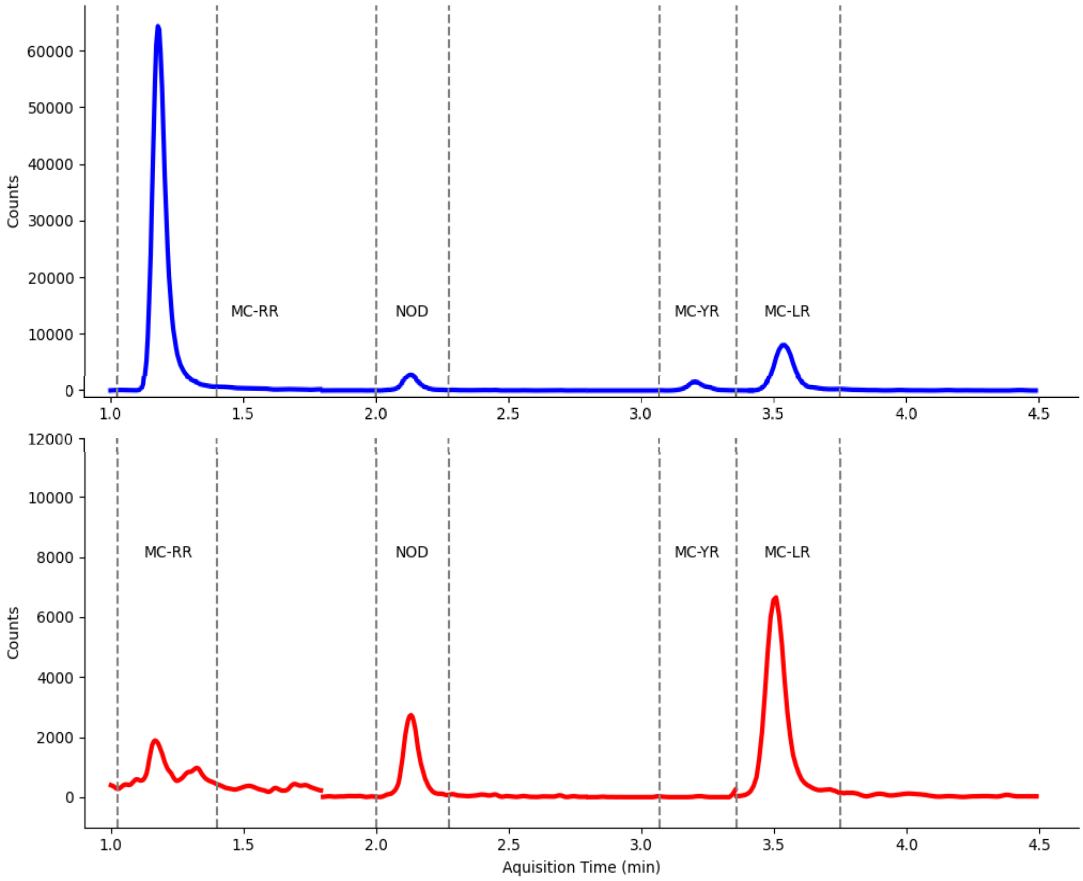
## 4.5 Supplementary information

**Supplementary Table 4.1** Concentrations of BMAA, 2,4-DAB and MC-LR in all mussels analysed. ND denotes non-detections, OR denotes over range and asterisks denote outliers which were removed prior to statistical analyses and visualisation

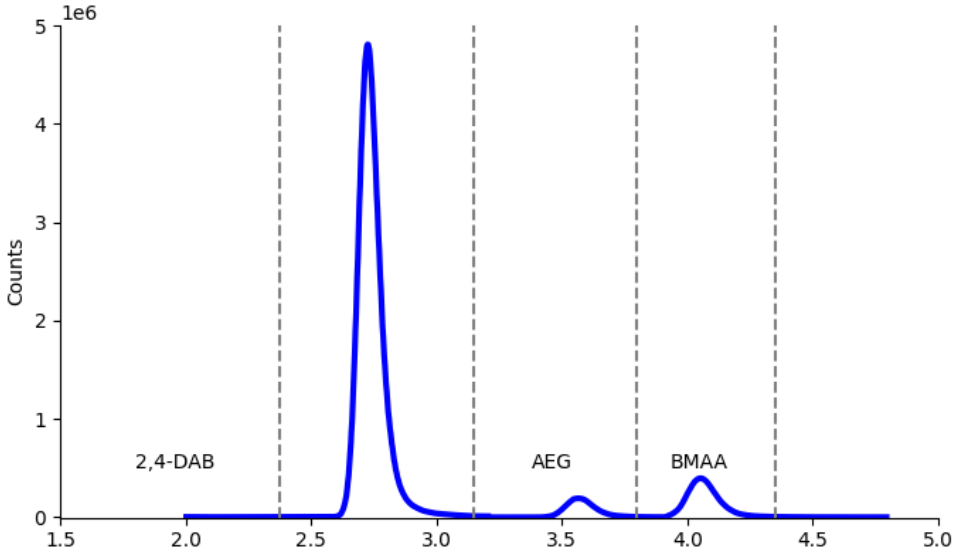
| Day | Repeat number | Soluble BMAA (ng/mussel) | Hydrolysed insoluble 2,4-DAB (ng/mussel) | Soluble 2,4-DAB (ng/mussel) | MC-LR (ng/mussel) |
|---|---|---|---|---|---|
| 0 | 1 | 1.356028 | 931.8453 | 12406.5 | ND |
| | 2 | ND | 4195.444 | OR | ND |
| | 3 | 5.398094 | 825.7682 | 17480.5 | ND |
| | 4 | 2.521115 | ND | 8437.302 | ND |
| | 5 | ND | 948.1756 | 18267.06 | ND |
| 3 | 1 | 2.850746 | ND | 10343.43 | 21.3013 |
| | 2 | 2.632621 | ND | 7724.21 | 29.06166 |
| | 3 | 2.468657 | ND | 13098.99 | ND |
| | 4 | 2.132262 | 960.7784 | 13728.97 | ND |
| | 5 | ND* | ND | 21743.47 | ND |
| 7 | 1 | 4.04662 | 1078.648 | 26137.73 | ND |
| | 2 | 1.897532 | ND | 8858.471 | ND |
| | 3 | 2.114958 | 1182.565 | 14945.47 | 923.7642 |
| | 4 | 3.693657 | 852.5726 | 16783.76 | ND |
| | 5 | ND | 1188.379 | OR | 19.44312 |
| 14 | 1 | ND | 2519.399 | 35403.47 | 24.76743 |
| | 2 | 2.415937 | 1008.334 | 17082.79 | 86.70252 |
| | 3 | 4.624667 | 1011.696 | 18155.66 | ND |
| | 4 | 2.308439 | 733.7089 | 11164.15 | 292.3731 |
| | 5 | 3.157564 | 1009.32 | 17016.6 | 50.15295 |

| | | | | | |
|---|---|---|---|---|---|
| 21 | 1 | 4.627423 | 1083.149 | 20719.39 | 197.5195 |
| | 2 | 4.043445 | 1144.174 | 11341.5 | 179.964 |
| | 3 | 1.513942 | 499.6455 | 7643.205 | 33.68624 |
| | 4 | 3.38598 | 3091.256 | 27905.57 | ND |
| | 5 | 3.329968 | ND | 13449.65 | 295.9184 |
| 28 | 1 | 5.174985 | 3296.647 | 30487.49 | 464.4378 |
| | 2 | 4.459187 | 1782.208 | 24026.74 | 87.63363 |
| | 3 | 0.401661 | 5364.051 | OR | 95.78824 |
| | 4 | 4.505826 | 1165.337 | 14487.75 | 141.9892 |
| | 5 | 3.507136 | 1446.733 | 18069.73 | 69.26072 |
| 42 | 1 | 3.678161 | 2117.844 | 32971.88 | 554.5343 |
| | 2 | 6.480357 | 2060.941 | 25915.34 | 3167.646 |
| | 3 | 3.808253 | 1564.05 | 15219.16 | 131.726 |
| | 4 | 7.334811 | 2148.179 | 13964.92 | 496.5047 |
| | 5 | 1.549564 | 1255.004 | 8180.829 | 182.6152 |
| 56 | 1 | ND | 12474.16 | OR | ND |
| | 2 | 4.013779 | 2472.954 | 34222.64 | 68.51161 |
| | 3 | 4.402105 | 1714.739 | 29668.65 | 334.4471 |
| | 4 | 5.875112 | 1355.906 | 27623.91 | 115.5387 |
| | 5 | 4.627903 | 1523.966 | 13132.81 | 122.3741 |
| 59 | 1 | 1.346399 | 330.34 | 21973.3 | 430.9504 |
| | 2 | 0.944582 | 131.4084 | 9895.208 | 71.42683 |
| | 3 | 2.268275 | 477.3726 | 36671.18 | ND |
| | 4 | 2.134887 | 398.6575 | 12908.81 | 46.25457 |
| | 5 | 0.496729 | 135.768 | 8015.369 | 31.09222 |
| | 1 | 1.404657 | 198.8893 | 10780.31 | 67.21305 |

| | | | | | |
|---|---|---|---|---|---|
| 63 | 2 | 1.483019 | 202.9375 | 13284.95 | 35.11664 |
| | 3 | 1.87377 | 304.6171 | 10540.22 | 2903.279 |
| | 4 | 1.943648 | 258.8237 | 11035.58 | 43.49892 |
| | 5 | 2.127884 | 420.909 | 13555.76 | 38.40235 |
| 70 | 1 | 2.21453 | 388.5581 | 10058.32 | 39.27372 |
| | 2 | 2.020048 | 348.6195 | 19083.85 | 57.06434 |
| | 3 | 2.042285 | 371.4237 | 19151.65 | 202.3563 |
| | 4 | 1.67175 | 328.2636 | 13797.64 | ND |
| | 5 | 0.751063 | ND | 6119.347 | ND |
| 77 | 1 | 1.496983 | ND | 13478.92 | 72.57303 |
| | 2 | 1.26694 | ND | 12077.75 | 64.15477 |
| | 3 | 1.630053 | ND | 13989.44 | ND |
| | 4 | 0.949408 | ND | 10339.34 | 22.14355 |
| | 5 | 0.249716 | ND | 1103.794 | 8.627475 |
| 88 | 1 | 3.096767 | 1217.001 | OR | 104.7284 |
| | 2 | 2.886098 | 548.9472 | 28103 | ND |
| | 3 | 1.919747 | 265.6715 | 23220.81 | ND |
| | 4 | 0.239249 | 38.73016 | 3363.185 | 12.71714 |
| | 5 | ND | 41.98569 | OR | 3.900573 |
| 112 | 1 | 2.747476 | 789.4935 | 17574.75 | ND |
| | 2 | 1.206979 | 208.3281 | 10636.42 | 26.27177 |
| | 3 | ND | 301.3062 | 13531.41 | ND |
| | 4 | 1.326299 | 299.9637 | 17833.42 | 48.18457 |
| | 5 | ND | 2650.644 | 31597.37 | 116.9386 |




**Supplementary Figure 4.1** Representative chromatograms of MCs and nodularin using the respective quantification MRM for each analyte. The blue line (above) is a chromatogram of a standard containing 10 ng/mL of each MC-RR, Nodularin (NOD), MC-YR and MC-LR. The red line (below) is of a representative sample with detectable concentrations of MC-LR and 10 ng/mL of nodularin as an internal standard.




**Supplementary Figure 4.2** Representative chromatogram of a standard containing 5ng/mL of AEG and BMAA and 50ng/mL of 2,4-DAB showing separation of each isomer.

# Chapter 5: *In situ* assessment of environmental toxin bioaccumulation in mussels


## Abstract

Cyanobacterial blooms are increasing in prevalence, and can have negative impacts on physical and chemical water quality through multiple mechanisms. One of these negative impacts is the production of toxins, such as the neurotoxin β-methylamino-L-alanine (BMAA), which has been associated with neurodegenerative disease through chronic exposure. Environmental exposure to BMAA is not currently regulated, partially due to inconsistencies in reporting its presence and the nature of its toxicity. Therefore new approaches are required to establish longer term monitoring programmes to better understand the proposed chronic toxicity mechanisms and peoples potential exposure to the toxin. One such monitoring solution is via utilising mussels as bioindicators of exposure to BMAA across a bloom, or multiple bloom events. In this study, Australian freshwater mussels, (*Velesunio ambiguus)* were translocated to two lakes in New South Wales that are prone to cyanobacterial blooms and held in mesh bags in surface waters for extended periods. The capacity of these mussels to uptake environmental BMAA and be used as monitoring tools was assessed. BMAA was detected in mussels from both lakes during periods of cyanobacterial blooms at concentrations up to 1.8 ng BMAA/g DW mussel, while no BMAA was detected in the mussels when there was no bloom present. This shows the feasibility of utilising mussels as bioindicators of BMAA in the environment.


## 5.1 Introduction

Cyanobacteria are photosynthetic prokaryotic organisms that are common in aquatic environments, where they are often referred to as blue-green algae.[5] Cyanobacterial blooms are of concern in freshwater ecosystems, as they can reduce the chemical and physical water quality.[13] One impact of cyanobacterial

blooms is the production of a range of toxic metabolites known as cyanotoxins.[151]

Some water authorities have implemented tiered risk alert systems for managing the health risks associated with cyanobacterial blooms in recreational waters, such as those used in Australia.[18] These systems use cyanobacterial biomass or cell counts as risk indicators, which simplify the monitoring process and allow for timely alerts without requiring complex toxin analyses. The tiered structure typically includes three levels: *Green*, indicating a small bloom and increased surveillance; *Amber*, signifying a moderate risk that warrants increased alertness; and *Red*, prompting immediate action due to risk to public health. This approach allows authorities to issue warnings and prevent public exposure to cyanobacterial toxins effectively. However, this method offers only a general estimate of health risks, which can lead to under- or overestimations of actual toxicity, as it lacks the specificity that direct toxin analyses provide.[19] While direct toxin analysis is more accurate, it is also costly, labor-intensive, and requires specialised expertise, limiting its routine use.

Many cyanotoxins, such as the hepatotoxic microcystins, are well understood and have implemented monitoring procedures, while others are emerging toxins of growing concern. [15, 218] One such toxin is the neurotoxic non-protein amino acid β-methylamino-L-alanine (BMAA). BMAA has been linked to high incidences of neurodegenerative disease globally,[86, 89, 219] with potential human exposure occurring through direct contact with contaminated water or bioaccumulation in plants and animals used as food sources.[54, 68, 70, 71, 181] However, its mechanisms of toxicity are debated in the literature.[45, 51, 101, 109] One obstacle to understanding BMAA's toxicity arises from its hypothesised chronic toxicity,[46] which is difficult to replicate *in vitro*.[46, 220] As a result, there are no levels of BMAA that can currently be considered "safe", which further hinders the inclusion of this toxin to water quality guidelines.

The chronic nature of BMAA as a toxin and its trace concentrations in the environment also necessitate a different monitoring approach than acute toxins so that its environmental presence is understood on the long-term scale that is

most relevant to chronic toxicity. Despite this, BMAA is commonly monitored through the analysis of single time-point grab sampling .[25, 26, 38, 75, 195]This is problematic, as BMAA concentrations in the environment have the capacity to change quickly,[171] and the primary concern is consistent, long-term exposure rather than acute toxin events. Therefore, single time-point sampling can't be used as representative BMAA concentrations over the duration of a bloom event and doing so can misinform by either under- or over-estimating the associated risk.

One potential alternative to single time-point grab samples is to use sentinel species as bioindicators of toxin levels over time. This would alleviate the issues individual samples have in lacking temporal information. Bioindicators have been used successfully for monitoring programs for several decades[129, 131] and are used for a range of water pollutants, including toxic metals,[128, 221] persistent organic pollutants,[222-224] and pharmaceuticals. [225-227]

The selection of sentinel species for this role is important. One of the most popular choices are filter-feeding bivalves. Bivalves are particularly suitable for this role as they are sessile and long-lived.[132] This enables them to be used for highly specific spatial information, and to be used for long periods of time. The other benefit comes from their filter-feeding nature, which facilitates the uptake of environmental pollutants from water.[132] The specific mussel species used also must be considered, as in environmental waters, only native or naturalised species are suitable to prevent the introduction of invasive species. Zebra mussels (*Dreissena polymorpha*)[228] and *Mytilus* species, such as blue mussels (*Mytilus edulis*),[137] are often used as bioindicators, but each has limitations. *Mytilus* are common globally,[137] but are limited to marine environments, while zebra mussels are freshwater species but are highly invasive and so are limited to areas where they are already present.[228]

BMAA has been measured in mussels,[55, 108, 118, 145, 146, 168, 169, 191] however, this has been primarily associated with a potential source of human exposure rather than as a way of monitoring environmental concentrations, as the mussels analysed were destined for human consumption. More recently, the uptake of

BMAA by mussels was assessed, including the uptake and depuration dynamics of BMAA from controlled exposure to BMAA-producing cyanobacteria,[191] as well as work assessing how free BMAA is taken up from water.[145, 146] Other experiments have looked at seasonal variations of BMAA concentrations in wild, local mussels, where a potential delay was noticed between the peak concentrations of BMAA present in the phytoplankton and the mussels.[76] However, using organisms native to the monitoring site presents known limitations for biomonitoring.[229, 230]. To date, there have been no temporal environmental exposure studies utilising translocated mussels. Research assessing the dynamics of BMAA uptake by mussels in the environment is needed to establish the suitability of mussels as bioindicators of this toxin.

A proposed mussel species to act as a bioindicator of BMAA in eastern Australia is the flood plain mussel (*Velesunio ambiguus*). This mussel has been used in previous controlled exposure experiments,[191] and found to have a strong positive trend between BMAA concentration and length of exposure to BMAA-producing cyanobacteria. *V. ambiggus* has a wide distribution across eastern Australia, including Queensland, New South Wales, Victoria and South Australia, and is particularly robust and long-lived,[231] making it a highly suitable bioindicator species.

The most suitable areas to assess mussels as bioindicators of environmental BMAA are in areas where cyanobacterial blooms regularly occur, such as the man-made lakes in rural New South Wales, Lake Wyangan and Mannus Lake. Mannus Lake regularly blooms at levels above guidelines for the recreational use of waterways [232-234]. Similarly, Lake Wyangan has a long history of regular cyanobacterial blooms,[235] and has had an increase in "red-alert" status in recent years.[236] Another point of interest for Lake Wyangan is the reported increased incidence of neurodegenerative disease in the nearby town of Griffith, which has been suspected to be linked to the regular cyanobacterial blooms of the surrounding waterways.[40] In both areas, these cyanobacterial blooms impact the public use of the waterways and have potential health impacts. This study, therefore, aims to assess the bioindicator potential of *V. ambiguus* mussels for

BMAA in the environment by assessing their uptake of the cyanotoxin when deployed into both Mannus Lake and Lake Wyangan.

## 5.2 Methods & materials

### 5.2.1 Reagents and materials

Mussels were purchased from Aquarium Industries Pty Ltd. (Epping Vic, Australia). LC-MS/MS analysis standards were L-BMAA hydrochloride (BMAA HCl, ≥97%, Merck, Bayswater Vic, Australia), L-2,4-diaminobutyric acid dihydrochloride (2,4-DAB 2HCl, ≥95%, Toronto Research Chemicals Inc., North York, ON, Canada) and N-(2-aminoethyl)-glycine (AEG, ≥97%, Toronto Research Chemicals Inc.) Internal standards were BMAA D3 (d3BMAA, ≥98%, Eurofins Technology, Dandenong South, Vic, Australia) and D-2,4-diaminobutyric-2,3,3,4,4-d5 acid dihydrochloride (d5DAB 2HCl, ≥66%, CDN Isotopes Pointe-Claire, QC, Canada). Reagents and solvents, including LC-MS/MS grade methanol and formic acid, HPLC-grade ethyl acetate and ammonium hydroxide, and analytical reagent grade trichloroacetic acid, hydrochloric acid, 1-propanol, 3-methylpyridine, chloroform propyl-chloroformate, and 2,2,4-trimethylpentane were purchased from Merck. Waters (Rydalmere, NSW, Australia) Oasis MCX, 3cc 60 mg, solid phase extraction cartridges were used. LC-MS/MS analysis was done with a Shimadzu Nexera UC UHPLC and a Shimadzu LCMS-8060 triple quadrupole MS (Rydalmere, NSW, Australia), with chromatographic separation by an Agilent Zorbax Eclipse Plus RRHD column and guard column (2.1 × 50 mm, 1.8 µm and 2.1 × 5 mm, 1.8 µm).

### 5.2.3 Environmental locations, deployment and sampling

HDPE net bags (approx. 20cmx20cm) containing 10 mussels each were deployed to the two sampling locations (Figure 5.1), Mannus Lake (-35°48', 147°58') and Lake Wyangan (-34°13', 146°1'). Samples were deployed to Lake Wyangan on 30/11/2020 and Mannus Lake on 02/11/2021. HDPE bags were suspended on a line in the water column, attached to a weight and float to ensure mussels were not exposed out of the water or in the sediment layer. Mussels were placed so that they would be near the surface of the water (approx. 30-60cm from the surface) to increase exposure to buoyant cyanobacteria. One cage containing n=10 mussels was collected at each time point. Whole mussels were stored frozen at -20 °C until processed for analysis.

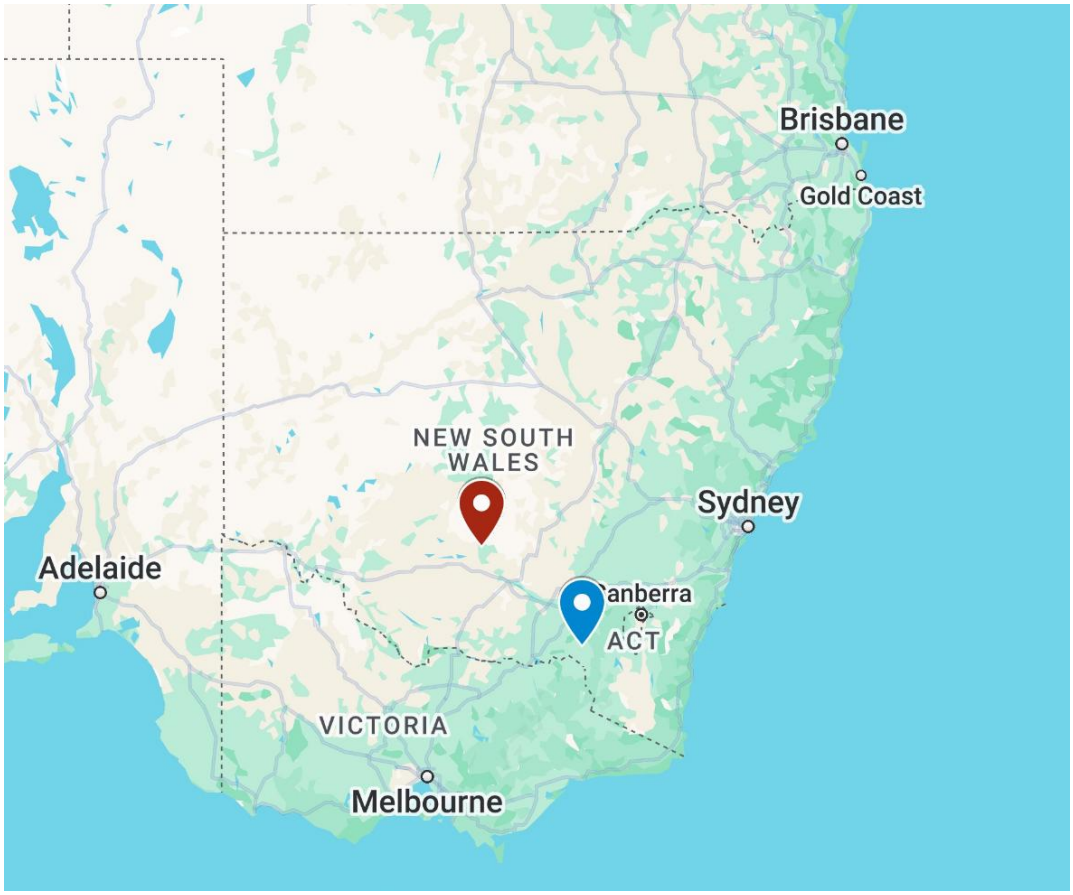



**Figure 5.1** Map showing locations of mussel deployment and sampling at Lake Wyangan (red) and Mannus Lake (blue).[237]

Samples collected from Mannus Lake were collected alongside routine monitoring of phytoplankton and water chemistry (fortnightly or monthly) as described in Facey et al.[233]

### 5.2.3 Sample preparation and LC-MS/MS analysis

Sample preparation was completed as described in Peters et al.[171] Five mussels from each sampled HDPE cage were shucked, and the tissue was freeze-dried, with wet weights and dry weights recorded for each sampled mussel. Dried mussel tissue (~50 mg) was lysed in 1 mL 10% trichloroacetic acid (TCA) with 20 µL of internal standard mix (500 ng/mL of d5DAB and d3BMAA) by probe sonication (50% intensity, twice for 1.5 mins), followed by overnight protein precipitation at 4°C. Samples were centrifuged (3000×*g* for 20 min), with the resulting supernatant combined with a further pellet wash of 400 µL 10% TCA. This fraction was freeze dried overnight (−80 °C, 0.1 mbar), while the protein pellet underwent vapour-phase protein hydrolysis (6M HCl, 110 °C for 16 h). 20 µL of internal standard mix was added to the hydrolysed pellet, and both fractions were reconstituted to a total volume of 400 µL. Samples were further extracted by Oasis MCX SPE cartridges. SPE cartridges were conditioned and equilibrated by subsequent additions of 1 mL 10% $NH_4OH$ in MeOH, 2 mL MeOH, and 1 mL 10 mM HCl. 200 µL of sample were loaded along with 800 µL 10 mM HCl, and were then washed with 1 mL 10 mM HCl, followed by 2 mL MeOH. An additional wash consisting of 1.5 mL 2% $NH_4OH$ in MeOH was done for the insoluble fraction. All samples were then eluted in 10% $NH_4OH$ in MeOH. The eluent was dried under nitrogen and then reconstituted in 100 µL 10mM HCl. Propyl-chloroformate (PCF) derivatisation was performed by the addition of 100 µL catalyst (20% 3-methylpyridine in 1-propanol), followed by 50 µL of derivatisation mix (propyl-chloroformate /chloroform/isooctane, 18%/72%/ 10%). The derivatised product was extracted by two additions of 100 µL ethyl acetate, which were transferred to LC vial inserts, dried under nitrogen and reconstituted in 100 µL 50% MeOH to match the LC starting mobile phase.

Standards of BMAA, AEG and 2,4-DAB were prepared, with BMAA and AEG concentrations in the range of 0.025–100 ng/mL, while 2,4-DAB were 10x higher at of 0.25–1000 ng/mL. All standards had 25 ng/mL of d3BMAA and d5DAB. Standards were derivatised with PCF using the same method as for the samples.

### 5.2.3 Analysis by LC-MS/MS

LC-MS/MS analysis was obtained using the validated method in Peters et al.[171] using a Shimadzu LCMS-8060. Separation was completed with a column temperature of 40 °C and flow rate of 0.8 mL/min. The mobile phase remained isocratic for the first 5 min, with 50 % A (ultrapure water with 0.1% formic acid) and 50% B (methanol with 0.1% formic acid). The mobile phase was then increased to 90% B until 7 min, and returned to 50% to equilibrate for a further 1.5 minutes. All samples were injected at 10 µL. The MS/MS was run in positive mode using multiple reaction monitoring.

## 5.3 Results & discussion

BMAA was detected in all mussel samples collected during cyanobacterial blooms from Mannus Lake (Figure 5.2) and Lake Wyangan (Figure 5.3). Only one sample date had no BMAA detected in any of the mussel replicates. This was the first collection date after deployment into Mannus Lake and was collected at a time when there was no cyanobacterial bloom present. Despite the historical occurrence of large blooms in Mannus Lake prior to deployment, only small, "green alert" blooms were observed during the year following deployment.

The unusually low phytoplankton biomass present in Mannus Lake during this year could have been influenced by the prolonged La Niña event, which saw Eastern Australia receiving increased rainfall, flooding, and cooler weather.[238] Increased rainfall can have several impacts on cyanobacteria presence, through impacting nutrient load, water temperatures, vertical stratification and flow rates.[239]

Larger scale blooms started to reoccur in Mannus Lake by late 2022, and subsequent samples taken during blooms of varying intensities showed

detectable BMAA concentrations. However, the concentration of BMAA did not correlate directly with cyanobacterial biomass, nor was there any noticeable "phase lag", as has been previously suggested in Kim et al.[76] where the peak concentrations of BMAA in mussels were delayed, occurring after the peak in cyanobacterial BMAA concentrations. The lack of correlation is particularly noticeable with the samples collected in February 2022 having the highest concentration of BMAA, despite being sampled during a time when there was relatively low cyanobacterial biomass present in Mannus Lake. One possible explanation for this is that the dominant species or genera of cyanobacteria present at that time point produced more BMAA than the cyanobacteria present during the other collection periods. *Microcystis* was present at the time the February 2022 samples were collected and stayed at approximately the same biomass or less (Supplementary Figure 5.1). Additionally, *Microcystis* was only present at one more of the sampling time points, in March 2023.

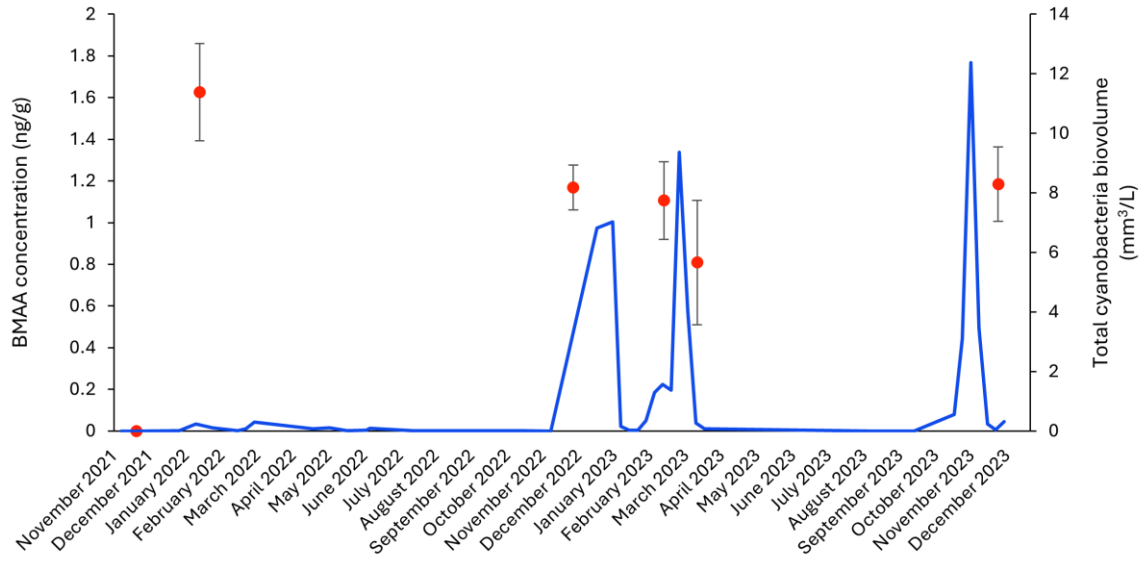



**Figure 5.2** Concentrations of BMAA in mussels (red) and the cyanobacteria biomass (blue) at Mannus Lake

This suggests that *Microcystis* could have produced higher BMAA concentrations than other cyanobacteria present. Previous studies have also shown *Microcystis* to contain elevated BMAA levels in Australian environmental samples, compared to other genera (Supplementary Figure 5.1) that were present in Mannus Lake for the duration of the experiment.[26] Additionally, *V.*

*ambiguus* have previously been shown to uptake BMAA from *Microcystis* by controlled exposure,[191] while this has not been proven for the other genera of cyanobacteria.

BMAA production by cyanobacteria can also be impacted by environmental conditions, such as nitrogen and phosphorus availability,[78, 79] which could also be impacting the amount of BMAA available to be taken up by the mussels at different time points. Further sampling and analysis, including sampling of cyanobacteria alongside the mussels so that cyanobacterial BMAA concentrations can also be assessed, would help with understanding what factors are driving the relatively similar BMAA concentrations in the mussels despite large changes in cyanobacteria biovolume. Additional factors contributing to the lack of correlation between BMAA concentrations in mussels and total cyanobacterial biovolume may include alternative sources of BMAA, or selective feeding patterns of the mussels. For example, diatoms and dinoflagellates, which were also present during this experiment (Supplementary Figure 5.2), are known to produce BMAA.[21, 22] Diatoms as a source of BMAA could explain the relatively high concentrations of BMAA in the mussels sampled in February 2022 (Figure 5.2), during which time there was also a spike in diatom biomass (Supplementary Figure 5.2). Alternatively, *V. Ambiguus* are known to alter their filter feeding under certain environmental conditions,[240] and can survive for long periods of inactivity,[241] allowing them to potentially avoid uptake of BMAA.

Unfortunately, for much of this study, due to the non-bloom conditions the cyanobacterial, or total phytoplankton, biomass was insufficient to allow for direct analysis of BMAA concentrations. However, this highlights a key advantage of using mussels as bioindicators. Mussels can accumulate BMAA from the water and even from small cyanobacterial biomass, making it possible to monitor BMAA levels through mussel sampling rather than relying on the collection of large amounts of water or cyanobacteria samples.

Unlike Mannus Lake, Lake Wyangan has had regular cyanobacteria blooms for the entire time mussels were deployed into the lake. However, sampling was

disrupted due to travel restrictions between 2020-2022 during the Covid-19 pandemic. Further issues arose from heavy rainfall events in 2022, which led to Lake Wyangan and the samples being inaccessible due to the high waters that resulted. Samples were later lost and not able to be recovered, likely as a result of the high water levels. This is a limitation of sampling caged, transplanted mussels rather than sampling from native populations.

Despite the difficulties faced with sampling procedures, BMAA was detected in all samples collected from Lake Wyangan. However, the concentrations were

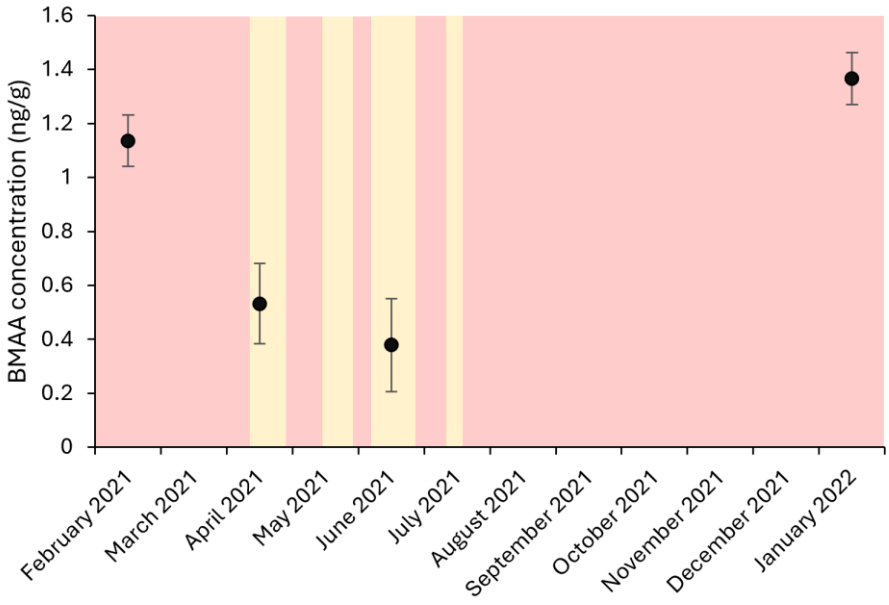



**Figure 5.3** Concentrations of BMAA in mussels and the cyanobacteria alert level (red or amber) at Lake Wyangan

mostly lower than those from Mannus Lake, despite the consistently high cyanobacterial biomass present at Lake Wyangan.

During the time mussels were deployed to, and being sampled from Lake Wyangan, the lake was consistently at red or amber level alert (Figure 5.3). The cyanobacterial bloom alert level was provided by the local council, but further information, such as dominant genus and total biomass were not made available. Mussels sampled during red alerts had significantly higher concentrations of BMAA than those sampled during amber alerts (t-test, $p < 0.05$). This trend shows the potential for *V. ambiguus* to uptake BMAA

from the environment during dense cyanobacterial blooms. However, more samples would be required to confirm this trend. It would also be beneficial, as with the samples from Mannus Lake, to have the cyanobacterial concentrations of BMAA to compare these time points to, enabling further validation of these mussels' ability to be used as bioindicators.

As mussels from both lakes had detectable BMAA, these findings show the ability of *V. ambiguus* mussels to uptake BMAA from the environment and show the potential for translocated mussels to act as bioindicators of exposure to BMAA. This also establishes a foundation for more robust temporal monitoring, which could facilitate a better understanding of the risk of chronic exposure to BMAA and potentially lead to the inclusion of BMAA in organised monitoring programs.

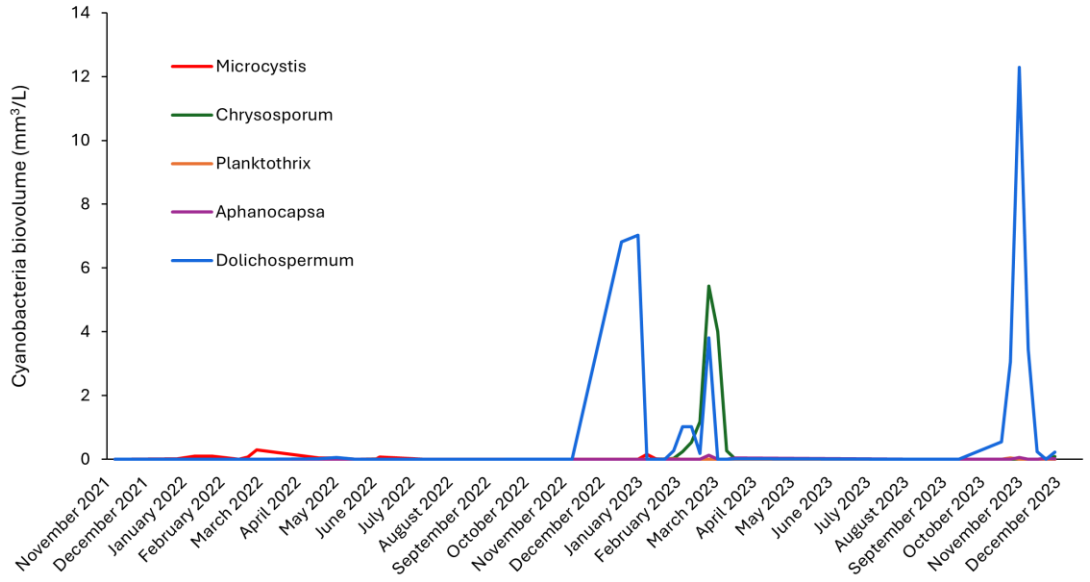
Future work should look to expanding this approach into more environments, including lakes that have sporadic cyanobacterial blooms and focus on assessing the concentrations of BMAA taken up by the mussels, as well as those present in the cyanobacteria, diatoms and water, where possible. Additional samples taken during periods when there are no cyanobacterial blooms would also be beneficial, to assess how the depuration mechanics work *in situ*, as in controlled exposure experiments, there was a rapid depuration. Additionally, work should be done with other mussel species to increase the geographical and ecological range of these bioindicator studies. Expansion to include the analysis of other contaminants of interest would also be beneficial to increase the value of information that is gained from these samples and potentially help this sampling method be more widely utilised.

## 5.4 Conclusions

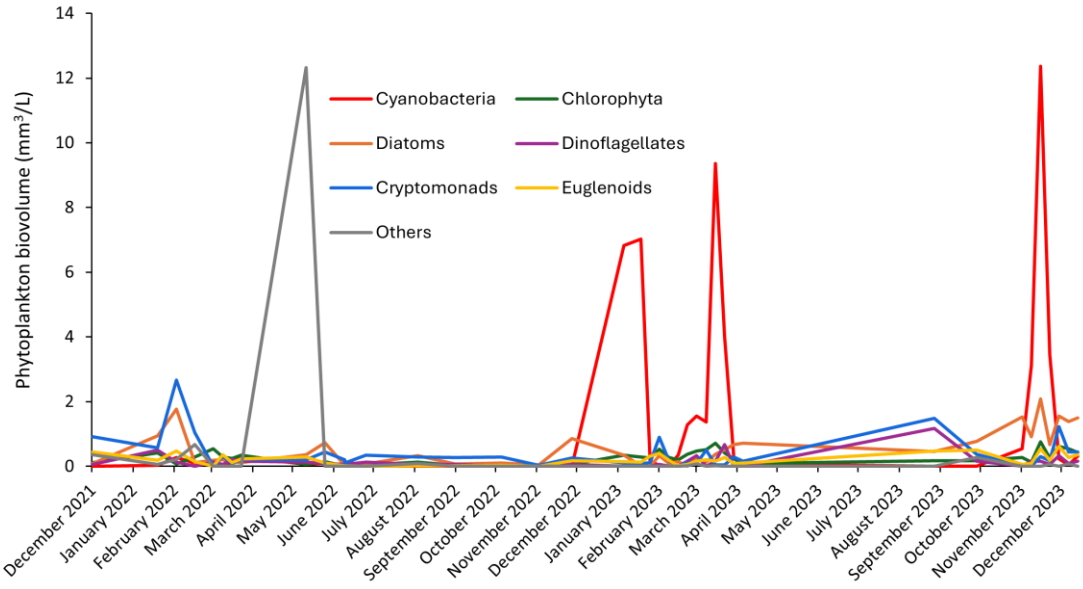
BMAA was detected in *V. ambiguus* mussels translocated into two lakes in New South Wales, Australia. Concentrations of BMAA were higher when cyanobacterial blooms were present, but concentrations did not always correlate with the scale of the bloom. In Mannus Lake, the concentrations of

BMAA were relatively stable when there were cyanobacteria present in the system, while in Lake Wyangan, BMAA concentrations were higher when there were dense (red alert) cyanobacteria blooms. This work shows the uptake of BMAA by *V. ambiguus* in the environment and establishes the ability of these mussels to be used as bioindicators of BMAA in the environment.

## 5.5 Supplementary Information




**Supplementary Figure 5.1** Biovolume of different genera of cyanobacteria present at Mannus Lake throughout the sampling period.




**Supplementary Figure 5.2** Biovolume of various phytoplankton groups present at Mannus Lake throughout the sampling period.

# Chapter 6: Conclusions and future directions

Cyanobacterial concentrations of BMAA have been shown to change rapidly within a single bloom event, showing the need for monitoring practices that include temporal information. The work in Chapter 2: shows rapidly decreasing concentrations of BMAA during the decline of a bloom event, with insoluble levels of BMAA dropping by over 50% in a space of four days, and dropping again by a similar amount in the following five days. Tracking of concentrations of BMAA across whole bloom events, with a larger sample size, would further the current state of knowledge. This could be used to inform the risk of exposure to BMAA during bloom events. An alternative to larger scale monitoring of cyanobacterial concentrations of BMAA is to use a sentinel species, such as filter feeding mussels, as a proxy of environmental levels.

Before mussels could be utilised as bioindicators of BMAA, a sensitive and selective method was required to detect BMAA in this complex matrix. The developed method had limits of detection 0.3 ng BMAA/g DW mussel and could confidently differentiate BMAA from its isomers, 2,4-DAB and AEG. The method used SPE, derivatisation with PCF and analysis by LC-MS/MS. As well as improvements in sensitivity from previous methods, the new method accurately corrected for matrix effects by including isotopically labelled internal standards for both 2,4-DAB and BMAA. This method was then used for the detection and quantification of BMAA in both lab-based and environmental experimentation that followed.

BMAA was found to accumulate in the Australian native freshwater mussel, *Velesunio ambiguus* in a controlled exposure experiment. Here, BMAA producing cyanobacteria was fed to *V. ambiguus*, and the resulting concentration of BMAA in the mussels correlated strongly to the length of exposure. This was trend was further validated by assessing the concentration of hepatotoxic microcystins, that were also produced by the exposure cyanobacteria, in the mussels. A similar trend was observed between the concentrations of BMAA and microcystins, strengthening the conclusion that

the increased concentrations were a result of exposure, as well as displaying the versatility for these mussels as bioindicators of cyanotoxins.

When these mussels were deployed into the field, BMAA concentrations were also detected when sampling occurred during periods of cyanobacterial blooms. However, for one of the two sites, Mannus Lake, there was no observed correlation between cyanobacteria biovolume and BMAA concentrations, potentially because of differences in BMAA production by the changing dominant phytoplankton. At the other site, Lake Wyangan, concentrations of BMAA were significantly higher during dense (red alert) blooms, than when the blooms were smaller (amber alert). This shows the potential for these mussels to be utilised as part of a temporal monitoring solution for BMAA exposure over time.

This thesis shows the need for temporal solutions to BMAA monitoring and provides a potential solution by utilising *V. ambiguus* mussels as bioindicators of BMAA concentrations over time.

Expanding the scope of these mussels as bioindicators to include other emerging contaminants, such as PFAS, could significantly expand the utility of this approach. Preliminary work has already detected PFAS in mussel tissue collected from field samples, while control mussels, not exposed to environmental waters, were free of PFAS (Appendix Figure 1). This suggests that native Australian mussels, such as *V. ambiguus*, could be an effective tool for PFAS monitoring. Broadening this approach to include toxic metals, persistent organic pollutants, and pharmaceuticals could greatly increase the value of biomonitoring efforts. Mussels have been used widely for monitoring various contaminants, but there is limited research on *V. ambiguus* in this capacity. Developing a deeper understanding of how these mussels accumulate and depurate a range of contaminants could enable multi-class toxin analysis and provide valuable temporal data on contaminant levels and exposure risks.

In addition to expanding the range of contaminants, it would be beneficial to assess the capacity of other mussel species to act as bioindicators of BMAA.

It is also important to consider the waterways that are being assessed and to choose suitable species local to that area. While *V. ambiguus* was selected for this research for its suitability to the regions of interest, its hardiness, long lifespan, and its availability, other species may be more suitable for other areas of Australia. For example, *V. ambiguus* are suited to slow flowing waterways, such as lakes and ponds, but not rivers,[242] so species such as *Alathyria jacksoni* could be more suited for bioindicators in Australian river systems.[242] Furthering research to include a wider range of bivalves would enable the most suitable bioindicator to be used for the system it is representing. This would be especially valuable for species already used in existing biomonitoring programs, such as the marine oysters (*Crassostrea virginica*) and mussels (*Mytilus spp*) used in the Mussel Watch Program.[243] By integrating BMAA analysis into these established frameworks, further information could be obtained from samples that are already routinely collected. Future studies should focus on evaluating the BMAA uptake dynamics in these species, as this would streamline monitoring efforts and provide comprehensive data on multiple environmental contaminants.

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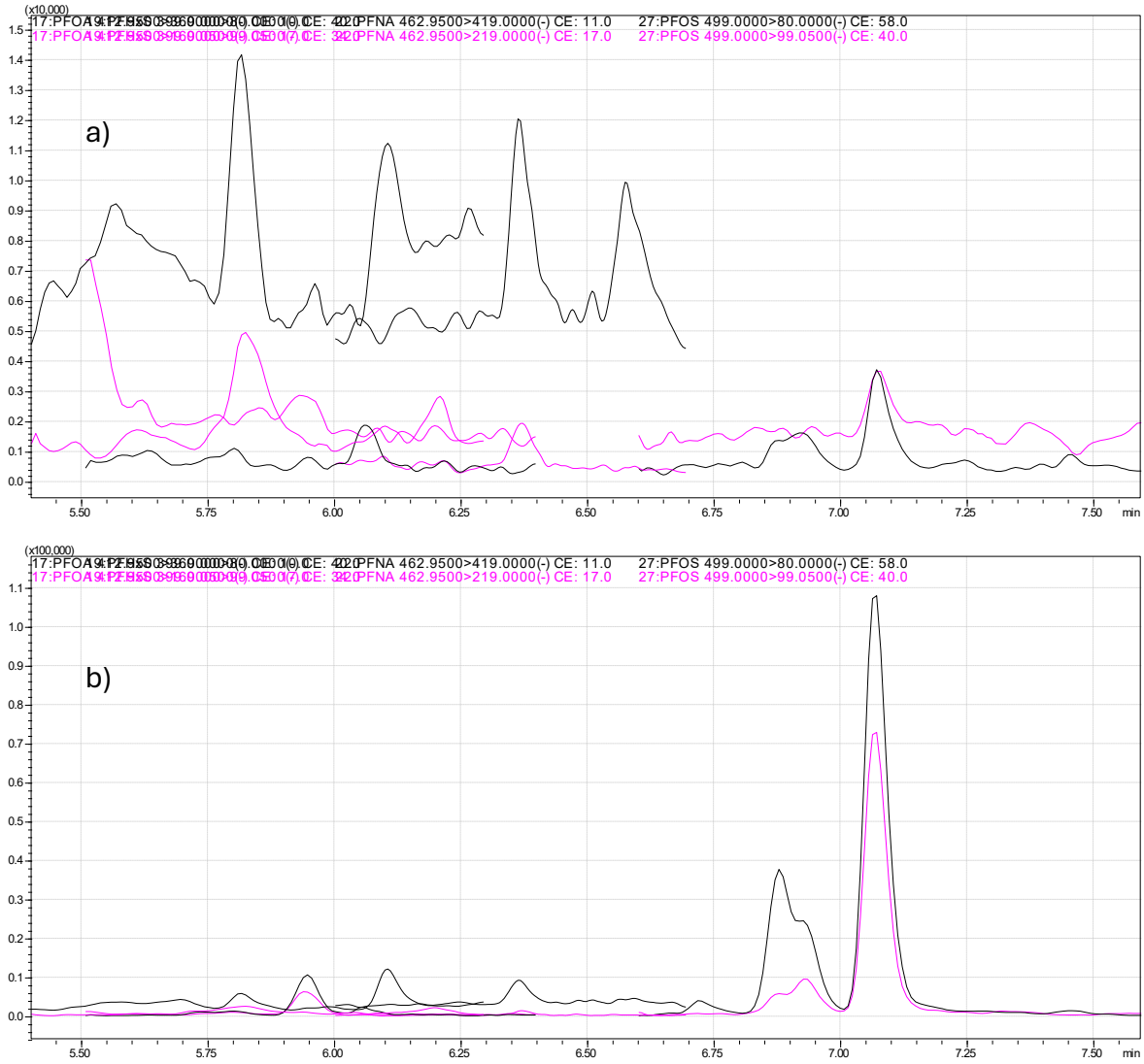
# Appendix




**Appendix Figure 1.** Chromatograms of mussel tissue with a) no environmental exposure (matrix blank) and b) environmental exposure showing PFAS compounds including PFHxS (Rt 5.95 min) and PFOS (Rt 7.1 min)