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# Detection of the suspected neurotoxin β-methylamino-L-alanine (BMAA) in cyanobacterial blooms from multiple water bodies in Eastern Australia

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ABSTRACT

The emerging toxin β-methylamino-L-alanine (BMAA) has been linked to the development of a number of neurodegenerative diseases in humans including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, and Parkinson's disease. BMAA has been found to be produced by a range of cyanobacteria, diatoms, and dinoflagellates worldwide, and is present in freshwater, saltwater, and terrestrial ecosystems. Surface scum samples were collected from waterways in rural and urban New South Wales, Australia and algal species identified. Reverse phase liquid chromatography-tandem mass spectrometry was used to analyse sixteen cyanobacterial scum for the presence of BMAA as well as its toxic structural isomer 2,4-diaminobutyric acid (2,4-DAB). BMAA was detected in ten of the samples analysed, and 2,4-DAB in all sixteen. The presence of these toxins in water used for agriculture raises concerns for public health and food security in Australia.



## 1. Introduction

Cyanobacteria are an ancient group of microorganisms that have colonised many terrestrial and aquatic environments. They produce a wide range of bioactive molecules, some of which are toxic to humans and animals. Cyanotoxins are a diverse range of molecules with mechanisms that include hepatotoxins such as microcystins, and neurotoxins such as saxitoxins and anatoxins (Aráoz et al., 2010; Funari and Testai, 2008; Rodgers et al., 2017; Van Apeldoorn et al., 2007). There is an increasing body of evidence to suggest that the non-protein amino acid (NPAA) β-Methylamino-L-alanine (BMAA) which is produced by cyanobacteria as well as diatoms and dinoflagellates (Esterhuizen and Downing, 2008; Jiang et al., 2014a; Jiang et al., 2013; Lage et al., 2014; Réveillon et al., 2016a), may also be toxic to humans and could contribute to the onset of neurodegenerative disease (Cox et al., 2017; Nunn, 2017). BMAA has been shown to trigger neurodegenerative processes in primate models; including the formation of β-amyloid plaques neurofibrillary tangles (NTFs) in vervet monkeys, and a number of neurological dysfunctions in macaques (Cox et al., 2016; Spencer et al., 1987).

Pablo et al. (2009) detected BMAA in the brains of neurodegenerative disease patients from North America, as well as the brains of sufferers of amyotrophic lateral sclerosis – Parkinson's dementia complex (ALS-PDC), an unusual neurodegenerative disease which was primarily observed in residents of the island of Guam in the 1960s. It is important to note that the Pablo study employed pre-column derivatisation with high pressure liquid chromatography – fluorescence detection (HPLC-FD) for analysis, post-hoc liquid chromatography tandem mass spectrometry (LC–MS/MS) was only used to confirm BMAA presence in positive samples. HPLC-FD has been shown to overestimate BMAA concentration (Faassen et al., 2012). A number studies employing alternative analysis techniques have not been successful in

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detecting BMAA in multiple matrices (Montine et al., 2005; Snyder et al., 2009), however, BMAA analysis has been shown to be prone to type II errors (Baker et al., 2017; Banack and Murch, 2018).

BMAA has been shown to be acutely toxic to primary neurological cells (Tan et al., 2017a,b), and two mechanisms have risen to prominence as potential causes for BMAA toxicity. In the presence of bicarbonate, BMAA has been shown to form a β-carbamate, which can act as a glutamate receptor agonist resulting in excitotoxicity (Chiu et al., 2015; Chiu et al., 2012, 2013; Zeevalk and Nicklas, 1989). BMAA has also been shown to bind to proteins, requiring hydrolysis to be liberated (Banack et al., 2007; Polsky et al., 1972). Evidence suggests that in eukaryotes this may occur through the misincorporation of BMAA into proteins during synthesis, possibly in place of serine (Dunlop et al., 2013; Glover et al., 2014; Main et al., 2017). The misincorporation of BMAA into proteins results in the formation of misfolded proteins within the lumen of the endoplasmic reticulum (ER), resulting in ER-stress and the activation of pro-apoptotic pathways as part of the unfolded protein response (UPR) (Main et al., 2016; Main and Rodgers, 2017; Okle et al., 2013; Shen et al., 2016). Both these mechanisms can result in a decline in function or death of neurological cells, potentially leading to symptoms observed in a number of neurodegenerative disorders.

Exposure to BMAA is of particular concern both because of its apparent ubiquity and its proposed ability to accumulate within plant and animal products (Andersson et al., 2018; Contardo-Jara et al., 2014,2018; Reveillon et al., 2015). BMAA has been found in numerous environments globally including aquatic and terrestrial ecosystems in North America (Banack et al., 2015b; Banack et al., 2014), Europe (Jiang et al., 2014b; Jonasson et al., 2010; Reveillon et al., 2015), Asia (Li et al., 2010,2016), South America (Johnson et al., 2008), Africa (Esterhuizen and Downing, 2008), and the Middle East (Chatziefthimiou et al., 2018; Cox et al., 2009). high concentrations of BMAA are often found in large fish or filter feeders (Hammerschlag et al., 2016; Reveillon et al., 2015; Réveillon et al., 2016b) increasing the risk of human exposure through diet. BMAA is also believed to be capable of spreading through aerosolisation, increasing the exposure risk for those who live near waterbodies, which have frequent cyanobacterial blooms (Banack et al., 2015b; Stommel et al., 2013). This risk is supported by epidemiological data collected from North America that shows an increased risk of ALS in people who live near water bodies with frequent cyanobacterial blooms (Bradley et al., 2017), as well as geospatial mapping data that shows increased frequency of ALS clusters near lakes with low water quality (Torbick et al., 2014, 2017).

To date, the detection and quantification of BMAA in Australian ecosystems has been limited. BMAA was detected in a single Australian isolate of *Cylindrospermopsis raciborskii* in an early survey of BMAA producing cyanobacteria (Cox et al., 2005). The apparent ubiquity of BMAA in other global ecosystems would suggest it is likely to be present in Australian cyanobacteria. Waterways such as those in the Murray-Darling basin in eastern Australia are of particular concern. The semi-arid environment, proximity of agricultural land, slow moving water, and high risk of eutrophication make these rivers susceptible to frequent large scale blooms (Baker and Humpage, 1994; Davis and Koop, 2006), some of which have exceeded 1000 km in length and have persisted for over a month (Al-Tebrineh et al., 2012; Bowling and Baker, 1996; Bowling et al., 2013). The frequency and scale of cyanobacterial blooms in eastern Australia, coupled with the multiple proposed exposure routes, suggest that people living in proximity to waterways may be regularly exposed to BMAA. Development of a robust testing program for Australian cyanobacterial blooms is critical to understanding the potential risk of exposure to BMAA in Australia.

This study aims to use established liquid chromatography – tandem mass spectrometry (LC–MS/MS) methods to analyse the presence of algal toxin BMAA and its isomer 2,4-Diaminobutanoic acid (2,4-DAB) in surface scum samples collected from waterways with cyanobacterial blooms in eastern Australia.

## 2. Experimental

### 2.1. Reagents

L-BMAA standard and analytical solvents and other reagents were purchased from Sigma Chemical Co. MO, USA unless otherwise stated. L-2,4-DAB and L-AEG was purchased from Toronto Research Chemicals ON, Canada. Δ-2,4-Diaminobutyric-2,3,3,4,4-$d_5$ (d5-2,4-DAB) was purchased from CDN isotopes, QC, Canada. EZ:faast HPLC free amino acid derivatisation kit was purchased from Phenomenex Inc. CA, USA.

### 2.2. Environmental samples

Surface scum samples for analysis were collected by dip sampling directly into the scum or by concentration through a 25 μm plankton net in 250 mL polyethylene sample bottles from a number of waterways in New South Wales (NSW) with cyanobacterial blooms. Because such samples are unsuitable for cell counting, addition 250 mL dip samples were collected from nearby clear water areas from 25 cm below the surface and preserved with Lugols iodine solution for quantitative analysis of major taxa present. Cyanobacterial species were identified and counted to species level for known toxigenic species, otherwise to genus level following the methods described by Hötzel and Croome (1999) using a calibrated Lund cell on a Zeiss Axiovert 35 inverted microscope (Carl Zeiss AG, Oberkochen, Germany) at 200× magnification to a minimum counting precision of ±20%. Counts were converted to biovolume using standard cell sizes determined for south-eastern Australia (Victorian Department of Sustainability and Environment, 2007). BMAA samples were stored at −20 °C until required then thawed on ice prior to analysis and pelleted through centrifugation for 20 min at 3000*g* and 4 °C after which water was removed and pellets freeze-dried overnight.

### 2.3. Sample processing and hydrolysis

Dry pellets were weighed, and a maximum of 50 mg transferred into fresh tubes with 1 mL of 10% trichloroacetic acid (TCA) in water, and probe sonicated at 80% power for one minute three times on ice. Samples were allowed to stand overnight at 4 °C, after which they were centrifuged for 10 min at 10,000*g*. Sample supernatant was transferred to a fresh labelled tube, and the pellet washed by resuspending and pelleting twice; with 300 μL 10% TCA in water, and once in 10% (v/v) TCA in acetone to reduce drying time. Supernatant from each wash was combined with the original supernatant and then placed in a centrifuge vacuum concentrator until dry. Protein pellets were transferred in to glass shell vials and placed in a centrifuge vacuum concentrator until dry. Dried supernatant was resuspended in 100 μL of 20 mM hydrochloric acid (HCl) and stored at −80 °C until needed.

Shell vials containing dried protein pellets were placed in a vacuum vial, and 1 mL of 6 M HCl added to the bottom of the vacuum vial. Air was removed from the vacuum vials using a vacuum pump, and nitrogen used to purge the vial, this was repeated three times. Samples were then placed overnight in a high temperature oven at 110 °C and hydrolysed in the gas phase of HCl. After hydrolysis, protein pellets were removed from the oven and briefly allowed to cool before being resuspended in 100 μL of 20 mM HCl. The supernatant and protein fractions were then