THE METABOLISM AND ENVIRONMENTAL FATE OF THE CYANOBACTERIAL NEUROTOXIN β-*N*-METHYLAMINO-L-ALANINE

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DECLARATION

I, Simoné Downing, 205000606, hereby declare that the thesis for the degree *Philosophiae Doctor* is my own work and that it has not previously been submitted for assessment or completion of any postgraduate qualification to another University or for another qualification.

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In the field of observation chance favours only the prepared mind.

- Louis Pasteur -

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SUMMARY

The neurotoxic amino acid β-*N*-methylamino-L-alanine (BMAA) is present in environmentally ubiquitous cyanobacteria and bioaccumulates and biomagnifies within the environment. The implication of BMAA in the development of neurodegenerative disease has raised concerns over the potential risk of human exposure to this neurotoxin, and has focussed attention on identifying possible routes of exposure that include direct contact with cyanobacteria and the ingestion of BMAA-containing plant and animal products.

The association of BMAA with cyanobacteria, and to some extent its environmental presence, have however been controversial, largely due to many reports claiming that BMAA is not present in cyanobacteria. Various controversies over the cyanobacteria-BMAA hypothesis were addressed in this work by investigating the metabolism and biosynthesis of BMAA in cyanobacteria, and the environmental fate of this amino acid. For this purpose current analytical methods used for BMAA quantification were evaluated and the most sensitive and accurate method was selected. Using this method, the cyanobacterial origin of BMAA was confirmed by showing the production of isotopically labelled BMAA from a labelled starting substrate. Investigation of metabolism of BMAA in the producing organisms revealed that the production of BMAA and its metabolism is closely associated with the cellular nitrogen status and potentially also with growth phase, and suggested a nitrogen-controlled regulatory role for BMAA in cyanobacteria. BMAA is produced under nitrogen deprivation and can induce cellular states such as chlorosis and quiescence, characteristic of those induced under nitrogen-limited conditions. Furthermore, a potential pathway for BMAA metabolism was identified in *Synechocystis* PCC6803, with a potential regulatory role for associated BMAA metabolites. BMAA is transaminated by glutamate synthase and the resulting BMAA keto acid may function as a response regulator, specifically under nitrogen-limited conditions.

The environmental fate of BMAA was evaluated by investigating the metabolism of this amino acid in model organisms. In the aquatic macrophyte, *Ceratophyllum demersum,* BMAA is taken up but not catabolised or excreted, but covalently modified and sequestered inside the cells. In freshwater mussels, BMAA is also taken up, not catabolised and only a small amount is excreted. Instead, BMAA appears to be covalently modified and sequestered inside the animal as a BMAA-derivative. This modification was reversible with the excretion of BMAA taking place during depuration.

This work showed that the environmental presence of BMAA is highly variable, not only because its biosynthesis in cyanobacteria is controlled by specific environmental conditions, but also because the amino acid is readily metabolised by other organisms. Therefore, as the neurotoxin may be present in a modified form, the inability the detect BMAA does not necessarily indicate its environmental absence.

Keywords: β-*N*-methylamino-L-alanine, cyanobacteria, metabolism, biosynthesis, environmental fate

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LIST OF ABBREVIATIONS

AA	amino acid
AD	Alzheimer's disease
AEG	*N*-2(aminoethyl)glycine
Ala	alanine
ALS/PDC	Amyotrophic Lateral Sclerosis / Parkinsonism Dementia Complex
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
amu	atomic mass unit
ANOVA	analysis of variance
AQC	6-aminoquinolyl-*N*-hydrosuccinimidyl carbamate
Arg	arginine
Asn	asparagine
Asp	aspartate
ATP	adenosine triphosphate
BEZ	Bezafibrate
BMAA	β-*N*-methylamino-L-alanine
BOAA	β-*N*-oxalylamino-L-alanine
cDNA	complementary DNA
CID	collision-induced dissociation
CNS	central nervous system
CSF	cerebral spinal fluid
C_T	threshold cycle
CYN	cylindrospermopsin
Cys	cysteine
DAB	diaminobutyric acid
DAP	diaminopropanoic acid
DAVA	D-aminovaleric acid
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DOM	dissolved organic matter
DON	6-diazo-5-oxo-L-norleucine
DPM	diaminopimelic acid
ESI	electron spray ionisation
Fd	ferredoxin
FD	fluorescent detection
FMOC	9-fluorenylmethyl chloroformate
GC	gas chromatography
Gln	glutamine
glnA	gene encoding type I GS
glnN	gene encoding type III GS
glnB	encodes PII protein
Gly	glycine
Glu	glutamate
GOGAT	glutamine oxoglutarate amidotransferase

GS	glutamine synthetase
HCl	hydrochloric acid
HILIC	hydrophobic interaction liquid chromatography
HPLC	high performance liquid chromatography
IR	infrared
Leu	leucine
LC	liquid chromatography
LOD	limit of detection
Lys	lysine
MC	microcystin
mGluR	metabotropic glutamate receptor
MS	mass spectrometry
MS/MS	triple quadrupole mass spectrometry
MSX	methionine sulfoximine
m/z	mass to charge ratio
NADH	nicotinamide adenine dinucleotide
nbl	non-bleaching genes
NMR	nuclear magnetic resonance
NMDA	*N*-methyl-D-aspartate
NTC	non-template control
NtcA	transcriptional regulator in nitrogen metabolism
OD	optical density
Orn	ornithine
P_{II}	nitrogen-controlled signalling protein
PCC	Pasteur Culture Collection
PCF	propyl chloroformate
pKa	symbol for acid dissociation
PCR	polymerase chain reaction
PD	Parkinson's disease
RNA	ribonucleic acid
RPLC	reversed phase liquid chromatography
ROS	reactive oxygen species
RSD	relative standard deviation
RT-PCR	real-time PCR
RubisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
Sar	sarcosine
Ser	serine
sig(ABCDE)	gene encoding *s*igma factors (A, B, C, D, E)
SPE	solid phase extraction
SRM	single reaction monitoring
TCA	trichloroacetic acid
T_R, RT	retention time
Trp	tryptophan
U	quantitative unit for enzymes
UPLC	ultra performance liquid chromatography
UV	ultra violet
Val	valine

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The non-proteinogenic amino acid β-*N*-methylamino-L-alanine (BMAA) was first discovered in seeds of the gymnosperm *Cycas micronesica* Hill (Vega and Bell, 1967), a discovery prompted by interest in the exceptionally high incidence of the neurodegenerative syndrome Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex (ALS/PDC) amongst the Chamorro people of Guam in the 10 years following the Second World War. ALS is an incurable progressive neurodegenerative disease characterised by muscle atrophy and spasticity. Ninety percent of all ALS cases are sporadic, having unknown cause (Chió *et al*, 2014). ALS-PDC syndrome combines symptoms of ALS, Parkinson's disease (PD) and Alzheimer's disease (AD).

For almost three and a half decades following its discovery BMAA was considered a cycad toxin. During this time its neurotoxicity was confirmed and characterised and substantial evidence accumulated that implicated this amino acid in the development of neurodegenerative disease. It was only in 2003 that BMAA was linked to environmentally ubiquitous cyanobacteria, when Cox *et al.* (2003) isolated the amino acid from the cyanobacterial species *Nostoc,* an endosymbiont of the coralloid roots of cycads. This link between BMAA and cyanobacteria was supported when the toxin was detected in 95% of all cyanobacterial strains tested, including endosymbiotic species and free living species from terrestrial, freshwater and marine habitats (Cox *et al*, 2005; Esterhuizen and Downing 2008; Metcalf *et al.,* 2008). The hypothesis that BMAA was produced not by cycads but by cyanobacteria refocused attention on BMAA as a neurotoxin, as the potential for human exposure was now far greater. Not only was the evidence of the presence of BMAA in cyanobacteria mounting, but also reports on the presence of the toxin within the environment and its bioaccumulation and biomagnification throughout food webs were progressively increasing. However, controversies arose over the detection and quantification of BMAA within cyanobacteria and other environmental samples. Some argued that BMAA was being misidentified and that analytical methods were overestimating the amount of BMAA in the environment.

A range of different analytical methods, including gas chromatography (GC), and reversed phase and hydrophilic interaction liquid chromatography (RPLC, HILIC), coupled to ultraviolet (UV), fluorescent (FD) or mass spectrometric (MS) detection of either derivatised or underivatised sample material, were being used to quantify BMAA. The large discrepancies in reported environmental BMAA concentrations, and the apparent absence of BMAA in some cyanobacterial cultures was thought to be largely as a result of the wide range of analytical methods employed, improper optimisation of such methods, and the lack of method comparisons and the validation of a single analytical method for BMAA analysis. Additionally, the cyanobacterial origin of BMAA was questioned because of the inability of many researchers to detect BMAA in cyanobacterial samples. This uncertainty was fuelled by the absence of any information on the biosynthesis of this amino acid and the very limited data on its metabolism within the environment. Whether there were aspects, other than differences in analytical sensitivity and accuracy, that could contribute to the large variations in reported environmental BMAA concentrations was therefore unknown.

Although there was evidence for the presence of BMAA in the environment, nothing was known about the fate of the amino acid in the environment, nor the factors that may modulate its production. Aspects that are important in gauging the extent of the risk of human exposure to BMAA, such as catabolism of, or metabolic chemical modifications to this neurotoxic amino acid by other organisms, or whether it could be released back into the environment following bioaccumulation, had been almost completely ignored. While research focusing on the mechanisms of BMAA neurotoxicity and its implication in neurodegenerative disease was advancing the development of an awareness of the potential human risk of exposure to this neurotoxin, the uncertainty over the origin of BMAA and the extent of human exposure to this toxin cast a shadow over the progress that was being made. It was essential to the progression of BMAA research that a reliable, robust and sensitive analytical method was identified that could be used in the quantification of BMAA in complex biological matrices. It was also necessary to confirm the cyanobacterial origin of BMAA, its biological role within the producing organisms and the factors that modulate its production, and the prevalence and persistence of BMAA within the environment.

1.2 LITERATURE REVIEW

1.2.1 β-*N*-methylamino-L-alanine: Discovery and link to neurodegenerative disease

In the eleven years that followed World War II, the leading cause of death in adults amongst the Chamorro people of the Western Pacific islands of Guam and Rota was a disease syndrome known to the local people as Lytico-bodig, and more commonly as Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex (ALS/PDC) (Whiting, 1963). This disease, which amongst the Chamorros had a 100 times higher incidence when compared to western populations (Kurland and Mulder, 1954), is an incurable neurodegenerative disease characterised by symptoms that are similar to those of ALS, PD and AD. Although environmental factors were always considered to play an important role in the development and the high incidence of this disease amongst the Chamorros, such factors were not formally characterised and the disease etiology remained uncertain. Early investigations suggested that the use of cycad plants (Cycadaceae), either as medicine or as food, was directly linked to the development of the disease. This cycad-ALS/PDC hypothesis was significantly strengthened when other locations were identified where the incidence of similar neurodegenerative disease complexes was significantly higher, and these locations were also characterised by the use of cycad plant material in food or medicinal preparations (Spencer *et al.*, 1987b; Spencer *et al.*, 1987c).

Based on the similarities that the disease ALS/PDC on Guam shared with the neurodegenerative disease lathyrism, investigators sought the neurotoxic amino acid implicated in the development of lathyrism, β-*N*-oxalylamino-L-alanine (BOAA), in the cycad seeds that the Chamorros were consuming. It was during this investigation that β-*N*-methylamino-L-alanine was discovered and characterised (Vega and Bell, 1967). In the years that followed the discovery of BMAA, research focused on the neurotoxicity of this "cycad" amino acid and the link between ALS/PDC of Guam and consumption of cycad products. The neurotoxicity of BMAA was first demonstrated in chicks by Bell and colleagues in 1967 (cited in Whiting, 1988) and various other studies followed, aimed at inducing ALS/PDC-like symptoms in animals fed either with cycad extracts or BMAA.

Although many studies reported acute neurological toxicity in various animals following the administration of BMAA (summarised in Karamyan and Speth, 2008 and in Chiu *et al.,* 2011), the high doses used in these studies and the acute or sub-acute nature of the toxicity was cause for some to dismiss the link between the exposure of the Chamorros to toxic amounts of BMAA via the consumption of cycad products, and the latent development of neurodegenerative disease. The inability to reproduce latent onset neurodegenerative symptoms in animal models as a result of exposure to relatively low doses of BMAA or cycad extracts led to the dismissal of the cycad-ALS/PDC hypothesis by the early 1990's.

Bioaccumulation of BMAA: ALS/PDC-BMAA hypothesis revisited

One of the main arguments against the cycad-ALS/PDC hypothesis was that the amounts of BMAA required to produce toxicity in animal models were considerably higher than the reported concentrations of BMAA in cycad seeds and in cycad-derived food products (Duncan *et al*, 1988; Duncan *et al*, 1990).

Investigation by Cox and Sacks (2002) into the presence of BMAA in other items of the Chamorro diet revealed the presence of BMAA in Flying Foxes (*Pteropus marrianus*) a species of fruit bat native to Guam and consumed as a delicacy by the Chamorros (Monson *et al*., 2003; Banack *et al.,* 2006). Cox and Sacks (2002) noted that Flying Foxes frequently fed on the fleshy sarcotesta of the cycad, *C. rumphii,* which contained high concentrations of BMAA (Cox and Sacks, 2002; Banack and Cox, 2003a), and hypothesised that BMAA was bioaccumulated within the Flying Foxes and that the Chamorros could be exposed to higher, more toxic concentrations of BMAA via regular consumption of these animals. This hypothesis was validated when BMAA was detected in free amino acid extracts of dried skin tissues of Flying Fox museum specimens (Banack and Cox 2003a). The concentration of BMAA detected in the bat skin specimens was up to a hundred fold higher than typical BMAA concentrations in the cycad seeds that the animals were consuming and considerably higher than previously reported BMAA concentrations in cycad seed flour (Banack and Cox 2003a). This report constituted the first evidence for the bioaccumulation and possible biomagnification of BMAA within a

food wed and provided new evidence in support of the cycad-ALS/PDC hypothesis by providing a route via which the Chamorros could be exposed to much higher doses of BMAA.

BMAA and cyanobacteria

While investigating the distribution of BMAA in the cycad, *C. micronesica*, Banack and Cox (2003b) noted the presence of BMAA in various tissues of the plant, including male and female reproductive tissues, leaves and coralloid roots. Although the presence of BMAA in the sarcotesta (frequently consumed by Flying Foxes and other foraging animals) was very important as it substantiated the BMAA biomagnification hypothesis of Cox and Sacks (2002), the presence of BMAA in the coralloid roots would prove to be of far greater importance.

BMAA was first associated with cyanobacteria following the reported correlation between the presence of BMAA and cyanobacterial endosymbionts in cycads (Banack and Cox, 2003b). In *C. micronesica* BMAA was only present in positively geotropic coralloid roots that were infected by endosymbiotic cyanobacteria and not in roots with normal morphology, and the amount of BMAA inside the coralloid roots corresponded to root maturity and the state of the endosymbiotic colonisation (Banack and Cox, 2003b; Cox *et al*., 2003). The highest concentrations of BMAA were present in coralloid roots with newly established actively growing cyanobacterial colonies, whereas coralloid roots with abundant but senescent cyanobacterial cells had substantially less BMAA, and no BMAA was detected in coralloid roots with no endosymbiotic cyanobacteria (Banack and Cox, 2003b). In 2003 Cox *et al*. confirmed the association between BMAA and cyanobacteria by detecting BMAA in axenic cultures of endosymbiotic cyanobacteria isolated from the coralloid roots of *C. micronesica.* The presence of BMAA in axenic cultures of endosymbiotic cyanobacteria isolated from non-cycad plants *Azolla filiculoides* and *Gunnera kauaiensis* (Cox *et al*., 2003) further confirmed the link between BMAA and cyanobacteria.

At this point, confirming the absence of BMAA in macrophytes devoid of cyanobacterial endosymbionts would have added value to the finding of Cox *et al*. (2003), and supported the notion that BMAA is a cyanobacterial and not a cycad metabolite.

Shortly following the report by Cox *et al*. (2003), Murch *et al*. (2004b) took the investigation of the cyanobacterial origin of BMAA one step further by looking at the BMAA content of protein extracts of axenic cyanobacterial (*Nostoc* sp.) cultures isolated from the coralloid roots of *C. micronesica.* The BMAA concentrations of the protein hydrolysates from these cyanobacterial cultures were 240 fold higher than the BMAA concentrations in free amino acid extracts of the same cyanobacterial isolates as previously reported by Cox *et al.* (2003). Murch *et al*. (2004b) confirmed the report by Polsky *et al.* (1972), that the amounts of BMAA present in the protein hydrolysates of cycad seeds and cycad seed flour were substantially greater than that previously reported in free amino acid fractions. These data not only substantiated the association of BMAA with cyanobacteria, but also supported the link between BMAA and ALS/PDC of Guam, as they provided evidence for exposure to much higher concentrations of BMAA than previously thought. However, Marler *et al.* (2010) argued against the cyanobacteria-BMAA hypothesis in stating that cycads could produce BMAA in the absence of endosymbiotic cyanobacteria. Marler *et al.* (2010) reported that the BMAA content of *C. micronesica* increased by 79% over nine months of seedling growth in the absence of endosymbioitic cyanobacteria. The conclusion that endosymbiotic cyanobacteria were not present in the seedlings was based only on the fact that original gametophytes were surface sterilised when they were harvested and that there were no coralloid roots present in the seedling at the time of analysis. Sensitive analytical tools, such as polymerase chain reaction (PCR) amplification of cyanobacteria-specific genes, were not used to confirm the absence of cyanobacteria in the cycads. Furthermore, only free BMAA was quantified using gas chromatography mass spectrometric (GC-MS) analysis of ethyl chloroformate derivatised BMAA. The authors therefore did not take into consideration that release of BMAA from a much larger protein-associated pool as described by Murch *et al*. (2004b) could have accounted for the increase in the free plant BMAA content.

In the years following the discovery of BMAA in both a free and protein-associated form in the symbiotic *Nostoc sp.,* the presence of BMAA was reported in other cyanobacterial species. Cox *et al*. (2005) reported the presence of free and/or protein-associated BMAA in 95% of all genera, and 97% of all strains, of free-living cyanobacteria tested and in 73% of all symbiotic *Nostoc* strains tested isolated from a wide range of taxonomically diverse lichens and plants. BMAA was present in cyanobacteria representative of all five cyanobacterial sections and diverse habitats, including freshwater, brackish and marine habitats, soil and hot springs. Banack *et al.* (2007) detected BMAA in a free-living marine *Nostoc* sp. using five different analytical methods and Esterhuizen and Downing (2008) using yet another analytical method, detected free and protein-associated BMAA in 96% of all strains tested. Esterhuizen and Downing (2008) reported that although BMAA was present in geographically and taxonomically diverse groups of cyanobacteria, there was no correlation between the amounts of BMAA present and taxonomy, or geographic origin. Metcalf *et al.* (2008) reported the presence of BMAA in all (n=12) tested samples, which comprised cyanobacterial bloom material, scum and mats, collected over 14 years from twelve waterbodies throughout the United Kingdom. The potential for the co-occurrence of cyanotoxins, which could potentiate BMAA toxicity, was also demonstrated by Metcalf *et al.* (2008). Protein-associated BMAA was also detected in seven genera of cyanobacteria sampled from 21 different lakes over a two-year period in the Netherlands (Lürling *et al.,* 2008).

These reports all supported the hypothesis that BMAA is a cyanobacterial metabolite and supported the suggestion made by Cox *et al*. (2005) that BMAA may be produced by all cyanobacterial species throughout diverse habitats. However, despite these data on the presence of BMAA in cyanobacteria, confirmation of the biosynthetic origin of BMAA from an identifiable raw material had yet to be published by the end of 2010.

BMAA and ALS/PDC

For more than three decades following the discovery of BMAA in cycads from Guam, the ALS/PDC-BMAA hypothesis had been rejected and revived several times. Even though more and more reports provided unique data supporting this hypothesis, substantiating evidence that would unify all these data and could link BMAA to ALS/PDC was still lacking. BMAA is neurotoxic (reviewed in Karamyan and Speth, 2008; and Chiu *et al.,* 2011), it is present in cyanobacteria and in various tissues of cycad plants, it bioaccumulates in fruit bats that feed on cycad seeds, it is present in cycad seed flour (and fruit bats) consumed by the Chamorros of Guam, and during the era in which fruit bats were frequently consumed by the Chamorros there was a considerably high incidence of ALS/PDC amongst these people. But this was not adequate evidence to confirm a role for BMAA in the development of this neurodegenerative disease syndrome. Questions that still clouded the BMAA-ALS/PDC hypothesis included: Can BMAA be linked directly to ALS/PDC? How were the Chamorros exposed to concentrations of BMAA reported to induce toxicity in animal models? How, when all animal model studies have shown acute or sub acute toxicity, could exposure to BMAA result in the latent onset of neurodegeneration in humans?

The direct link between BMAA and ALS/PDC was strongly validated when BMAA was detected in brain tissues of patients who had died from this disease, but not in patients who had died of causes unrelated to sporadic neurodegenerative disease (Cox *et al*., 2003; Murch *et al.*, 2004a; Murch *et al.*, 2004b). Cox *et al*. (2003) and Murch *et al.* (2004a) reported the presence of free, and free and protein-associated BMAA, respectively, in the brain tissues of six Chamorros who had died from ALS/PDC and in two Canadian patients who had died from AD, but not in the brains of thirteen patients who had died from causes unrelated to neurodegenerative disease. Murch *et al.* (2004a) also reported that BMAA was found in one of two Chamorros who had died from asymptomatic causes. Overall, when present in brain tissues, the amounts of protein-associated BMAA were 10-100 fold more compared to those detected in free amino acid extracts (Murch *et al*., 2004a). These data not only directly linked BMAA to ALS/PDC and AD, but also showed that BMAA may be implicated in non-sporadic

neurodegenerative disease in non-Chamorros, which suggested that the causative or contributing agent was not unique to the Guam ecosystem or culture. These findings were challenged by studies that reported the failure to detect BMAA in brain tissues of individuals who had died from ALS/PDC. Montine *et al.* (2005) analysed free amino acid extracts of brain tissues of 20 individuals from Guam and the United States Pacific Northwest, including individuals diagnosed with AD and PD and individuals showing no symptoms of neurodegeneration. Using a previously validated method (Kisby *et al.,* 1988), with a reported limit of detection (LOD) of 1 pmol of BMAA, that employed RPLC and fluorescent detection of 9-fluorenylmethyl chloroformate (FMOC) derivatised samples, the authors reported the absence of BMAA in all of the brain tissues analysed. For reasons unknown, the protein-associated fractions were not analysed. Considering the substantial variations between free and protein associated BMAA in brain tissues reported by Murch *et al*. (2004a), the report by Montine *et al.* (2005) seems premature and incomplete. Chen *et al.* (2002) and Wilson and Shaw (2006) also challenged the link between BMAA and ALS/PDC, suggesting that some data used to support this theory were false or that there was simply not enough evidence to support the BMAA-ALS/PDC hypothesis. In 2009 an independent study reported the detection of free and/or protein-associated BMAA in brain tissues of individuals who had died with ALS and AD (Pablo *et al.,* 2009). This blinded study was performed independently on brain tissues from residents of the United States of America using the same method as previous described by Cox *et al*. (2003) and Murch *et al* (2004a). Pablo *et al.* (2009) reported the presence of BMAA in 100% of all brain tissues of individuals who had died with ALS (13 samples) or AD (12 samples) and the absence of BMAA in 8 of 9 brain tissues of individuals who had died with the non-sporadic neurodegenerative disease, Huntington's disease, and 10 out of 12 individuals who died with no neurodegenerative symptoms. This independent study, with a relatively larger sample size, strengthened the hypothesised BMAA-ALS/PDC link and the fact that the link between BMAA and neurodegenerative disease is independent of Guam and/or contact with cycads or consumption of cycad products or fruit bats.

As previously stated, Murch *et al*. (2004b) reported that considerably higher concentrations of BMAA were present in a protein-associated form compared to that present as a free cellular amino acid. This held true not only in brain tissues but also in various other samples tested; cyanobacteria, cycad tissue and cycad seed flour. These findings impacted on the BMAA-ALS/PDC hypothesis in that they showed that the amount of BMAA that the Chamorros were being exposed to through the consumption of cycad food products or Flying Foxes was considerably higher than previously thought, and that the criticised unrealistic concentrations of BMAA used in toxicological studies (Spencer *et al.,* 1987a) were in fact representative of the Guam food web. More importantly, these findings suggested a mechanism through which exposure to BMAA could result in the latent onset of neurodegenerative disease. Spencer *et al*. (1991) proposed that, because there may be a long period between exposure to cycad toxins (such as BMAA) and the onset of neurodegenerative disease, the causative agent my be a 'slow' toxin, which causes the slow, irreversible degeneration of neurons. Murch *et al*. (2004b) postulated that the protein-associated BMAA pool may act as a toxin reservoir from which, during protein turnover, BMAA is slowly released over a long period of time, resulting in the gradual degeneration of neurons and the latent onset of neurodegenerative disease.

Research in the early 2000's addressed key aspects of the BMAA-ALS/PDC hypothesis, and, although data from some investigations did not support this hypothesis, data from many other studies did support the hypothesised implication of BMAA in ALS/PDC and the associated hypothesis that cyanobacteria could serve as a universal link between humans and BMAA exposure.

Mechanisms of BMAA toxicity

Data from several independent studies on the toxicity of BMAA in animal tissue suggest that BMAA may elicit neurotoxicity via various mechanisms (reviewed in Chiu *et al.,* 2011), including: protein misfolding and aggregation due to possible BMAA misincorporation into proteins (Rodgers and Dunlop, 2011; Dunlop *et al.,* 2013), and over excitation of motor neurons and the induction of oxidative stress.

BMAA β-carbamate adducts that form in the presence of bicarbonate at physiological concentrations and pH (Weiss and Choi, 1988; Mroz 1989, Nunn and O'Brien, 1989; Weiss *et al*., 1989) resemble the excitatory amino acids, glutamate and aspartate, and can compete with these ligands for binding to glutamate receptors, such as *N*-methyl-D-aspartate (NMDA) receptors (Ross *et al*., 1987; Nunn and O'Brien, 1989; Allen *et al*., 1995; Lobner *et al*., 2007; Lobner *et al.*, 2009) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Weiss and Choi, 1988, Weiss *et al.,* 1989; Rao *et al.,* 2006), as well as other ionotropic and metabotropic glutamate receptors (Copani *et al*., 1991; Meldrum and Garthwaite*,* 1990; Lopicic *et al.,* 2009; Cucchiaroni *et al.,* 2010). Binding of BMAA to such receptors results in the disruption of the equilibrium of sodium, potassium and calcium ions, causing membrane depolarisation and permeability, and neuronal damage.

BMAA inhibits the Xc^- cystine/glutamate antiporter system (Liu *et al*., 2009), a transporter system that mediates the cross-plasma membrane exchange of glutamate and cystine thereby maintaining the intercellular cystine balance required for glutathione production and protection against oxidative stress (Bridges *et al.,* 2012). Disruption of the cellular calcium ion (Ca^{2+}) balance has a cascading effect resulting in a decrease in cell viability. An increase in intracellular Ca^{2+} results in mitochondrial membrane disruption and consequently in an increase in the production of cellular reactive oxygen species (ROS) leading to oxidative stress (Lobner *et al*., 2007; Rao *et al.,* 2006; Cucchiaroni *et al.,* 2010). BMAA also causes oxidative stress via the inhibition of the Xc^- cystine/glutamate antiporter system, which also results in the release of glutamate that subsequently causes excitotoxicity via over excitation of the metabotropic glutamate receptor, mGluR5 (Liu *et al*., 2009).

Apart from excitotoxicity and oxidative stress, it has also been suggested that BMAA may be misincorporated into cellular protein, resulting in protein aggregation and misfolding (Rodgers and Dunlop, 2011; Dunlop *et al.,* 2013).

A greater knowledge of the mechanisms of BMAA toxicity is necessary to fully understand the role of BMAA in the development of neurodegenerative disease. Although evidence suggests that BMAA toxicity is a function of various different mechanisms, the direct link between human exposure to this neurotoxin and latent onset neurodegeneration has yet to be proven.

1.2.2 Analytical controversies over detection and quantification of β-*N*-methylamino-L-alanine

One of the most common arguments against the cyanobacteria-BMAA-ALS/PDC hypothesis was based on the discrepancies observed in analytical results of BMAA in biological samples. Since the discovery of BMAA (Vega and Bell, 1967), discrepancies in the presence of BMAA in environmental samples and in the presence/absence of BMAA in particular organisms, and large variations in the amounts of BMAA reported in various biological samples, became evident. In trying to explain these discrepancies, some attributed them to differences in the sensitivity or accuracy of analytical methods used (Krüger *et al.,* 2010; Faassen *et al.,* 2012). Although attempts have been made to evaluate and/or compare the suitability of analytical methods for quantification and/or detection of BMAA (Beckman *et al.,* 2007; Cohen, 2012; Faassen *et al*., 2012), to date there is still no general consensus amongst investigators as to the best method for BMAA analysis in complex biological matrices.

The development of analytical methods for the detection and quantification of BMAA was relatively slow during the first three decades following its discovery. Driven by the first reports of BMAA in human brain tissue and in cyanobacteria, attempts to develop unique analytical methods and efforts to optimise or revise existing methods intensified in the early 2000's.

In 1967, Vega and Bell used a combination of paper chromatography and ionophoresis together with cation exchange, reaction with ninhydrin and, infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy. Polsky *et al.* (1972) successfully used an automatic amino acid analyser to quantitatively analyse the BMAA content of

hydrolysed and unhydrolysed cycad extracts. Mass spectrometry, elemental analysis, ^{1}H-NMR, high voltage electrophoresis and thin layer chromatography were used by Reece and Nunn (1988) to confirm the authenticity of synthesised radiolabelled BMAA. In all early studies, in which mostly non-instrument based analytical methods were used for BMAA analysis, the authenticity of reported BMAA was rarely questioned. Concern over the possibility of BMAA being an artefact of acid hydrolysis, was confidently dismissed based on the fact that BMAA is present in non-hydrolysed samples (Polsky *et al.,* 1972). The use of these early methods demonstrated that BMAA was highly stable under acidic conditions and that separation of BMAA from its isomer, 2,4-diaminobutyric acid (2,4-DAB) could be achieved using relatively crude methods, such as ionophoresis.

The first method employing pre-column derivatisation of BMAA was published in 1988 (Kisby *et al*.). In this method the fluorogenic agent FMOC was used to derivatise amino acids prior to chromatographic separation (RPLC) and optical detection of fluorescent amino acid derivatives. Quantification of amino acid derivatives was based on the internal amino acid standard, D-aminovaleric acid (DAVA). Although separation of BMAA from biologically relevant isomers was not considered by Kisby *et al*. (1988), the reported method did successfully separate BMAA from 17 other amino acids including gamma-aminobutyric acid, and was able to quantify BMAA in cycad seed extracts, brain tissue, serum and cerebrospinal fluid (CSF) of rats and non-human primates exposed to BMAA. This method was adapted by Montine *et al.* (2005) for analysis of BMAA in brain tissue of individuals who had died of either ALS or AD and control patients. The only modification was to the elution gradient used, and resulted in a longer elution time (more than double that was originally used by Kisby *et al.* (1988)). However, no BMAA was detected in any of the samples analysed by Montine *et al.* (2005). The LOD of these two methods were comparable (0.6 pmol on column, Kisby *et al*., 1988, and 1.0 pmol on column, Montine *et al*., 2005), but a hundred fold more sensitive than the method used by Murch *et al.*, (2004a) by which BMAA was detected in brain tissue. This highlights the fact that there are potentially many aspects that influence the accuracy and reproducibility of BMAA analytical methods, including sample origin, sample preparation, chromatographic separation and mode of detection, instrument variability, and the precision of the individual hand.

Following the first report on the presence of BMAA in cyanobacteria, most subsequent studies reporting positive results for BMAA in cyanobacteria, employed the same method: LC separation with either fluorescent or MS detection of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatised amino acids (Cohen, 2012). However, in 2008 Rosén and Hellenäs attempted, unsuccessfully, to detect BMAA in cyanobacteria using HILIC with MS detection, a method that did not employ pre-column derivatisation. Their failure to detect underivatised BMAA in cyanobacteria using this method, brought into question the cyanobacterial origin of this amino acid and the validity of previously reported data. This uncertainty was fuelled by the fact that the same method that failed to detect BMAA in cyanobacteria positively identified the neurotoxin in cycad extracts (Rosén and Hellenäs, 2008). In the same year, yet another unique method, using ethyl-chloroformate derivatisation of amino acids with GS-MS, was employed to analyse the BMAA content of various freshwater cyanobacterial species (Esterhuizen and Downing, 2008). The presence of free and/or protein associated BMAA was reported in the majority of strains tested. This method, using an amino acid analysis kit (EZ:faast™), offered a high level of reproducibility and included pre-derivatisation sample concentration on a sorbent medium resulting in increased sensitivity of amino acid quantification in a complex matrix. However, despite these advantages, this method was not widely adopted for analysis of BMAA in environmental samples.

Although a variety of methods have been employed, successfully and unsuccessfully, in the analysis of BMAA in biological matrices, only a few methods have been used routinely. These methods are distinct in pre-analysis sample preparation, and the type of chromatographic separation and/or detection used, with the majority of these methods incorporating pre-column derivatisation with AQC or propyl chloroformate (PCF), followed by either LC-FD or LC-MS. The other routinely used method does not include derivatisation, but employs HILIC separation and mass spectrometric detection of underivatised analytes. Generally, when analysing low molecular weight molecules, such as amino acids, the use of derivatisation increases the sensitivity of analysis, as, by increasing the molecular weight of the analyte, interference by the sample matrix is reduced (Li *et al*., 2010; Cohen, 2012). In the majority of studies employing HILIC-MS

analysis of BMAA, the neurotoxin was commonly not detected in cyanobacterial samples, but was detected in cycad extracts (Kubo *et al.,* 2008; Rosén and Hellenäs, 2008; Faassen *et al.,* 2009; Bidigare *et al.,* 2009; Li *et al*., 2010; Krüger *et al*., 2010; Li *et al.,* 2012; Faassen *et al.,* 2012; Combes *et al.,* 2013). Contrastingly, in the majority of studies using LC-MS analysis of derivatised amino acid extracts, BMAA was detected in cyanobacteria and in cycad material, but, occasionally, the reported BMAA content of cyanobacterial extracts varied considerably (Banack *et al*., 2007; Metcalf *et al.,* 2008; Johnson *et al.,* 2008; Esterhuizen and Downing, 2008; Cox *et al.,* 2009; Eriksson *et al.,* 2009; Spáčil *et al.,* 2010). Consequently, the failure to detect BMAA and the large variations in reported BMAA concentrations raised questions over the authenticity and accuracy of these reports. Despite the obvious issue of method suitability, this highlighted the importance of considering other possible causes of these discrepancies. The inability to detect BMAA in cyanobacterial extracts does not automatically indicate the absence of the amino acid, nor the unsuitability or inaccuracy of the method employed, but may, as suggested by Li *et al*. (2010) indicate that the presence of BMAA in cyanobacteria is modulated by certain growth conditions, and that BMAA is not present in cyanobacteria during exponential growth under laboratory conditions. Similarly, but perhaps more importantly from the perspective of potential for human exposure, the lack of BMAA in other biological samples is not immediately indicative of the absence of the neurotoxin in a particular environment, but may suggest that the amino acid is metabolised by organisms within that environment.

Considering that there are various non-analytical factors that could influence the detection/presence of BMAA, most importantly the potential metabolism of BMAA in the environment and probable variation in biosynthesis and/or environmental content as a function of environmental parameters, it is imperative that a reproducible and sensitive method for accurate quantification of BMAA in complex biological matrices is selected, so that, when investigating these non-analytical factors, variation in BMAA content can not be attributed to the analytical method used.

1.2.3 Chemical reactivity, biological role, metabolism and biosynthesis of β-*N*-methylamino-L-alanine

Chemical reactivity

BMAA, with a molecular weight of 118.13 $g.mol^{-1}$, is a polar, hydrophilic amino acid with two functional amino groups. The 2-amino groups of most amino acids, including the proteinogenic diamino acids, arginine and lysine, contain a net positive charge at physiological pH values (7.00-7.40), and are consequently relatively unreactive and require enzyme-catalysed deprotonation for bond formation (Clayden *et al.*, 2001). One aspect that makes the diamino acid BMAA an usual amino acid, is that its 2-amino group has no net charge at physiological pH, making it more reactive than other proteinogenic diamino acids, a trait that could contribute to its toxicity (Nunn and Ponnusamy, 2009). The 2-amino group of BMAA has a relatively low pK_a value (pK_2=6.50) (Vega and Bell, 1967; Nunn and Ponnusamy, 2009), and the close proximity of the two amino groups results in interactions between them that effect their net charge. Consequently, at physiological pH a positive charge is shared by the two amino groups and is cancelled out by the negative charge of the carboxyl moiety, resulting in a net neutral charge of BMAA (Figure 1.1).

Weiss and Choi (1988) proposed the first possible chemical modification of BMAA. By the late 1980's, although the neurotoxicity, and more specifically the excitoxicity (Olney *et al*., 1976; Nunn *et al*., 1987; Ross *et al*., 1987) of BMAA had been demonstrated in various *in vivo* models (Vega *et al.,* 1968; Polsky *et al*., 1972; Ross *et al.,* 1989; Spencer *et al.,* 1987a), the precise mechanisms of toxicity were still unclear. BMAA does not have the terminal acidic moiety characteristic to other excitatory amino acids (Figure 1.2) such as glutamate and aspartate (Weiss *et al*., 1989). In order to understand how BMAA could exert excitotoxicity, Weiss and Choi (1988), following the observation that BMAA excitotoxicity of glutamate receptors is dependent on the presence of physiological concentrations of bicarbonate, proposed that the beta amine of BMAA reacts with bicarbonate to form a structure that more closely resembles an excitatory amino acid.

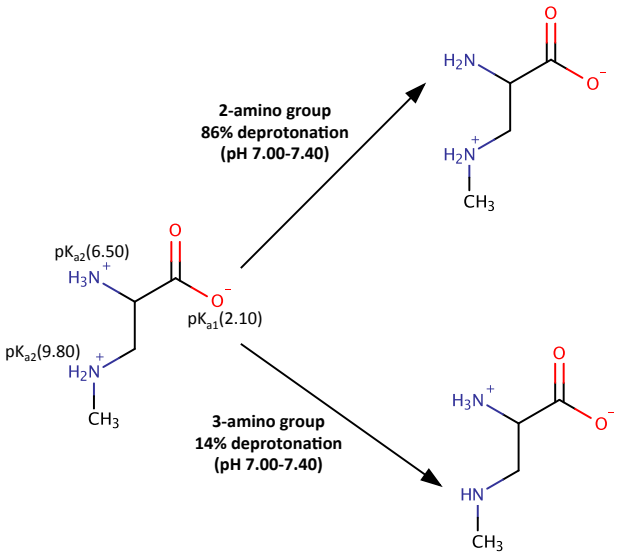

Figure 1.1: The protonation of BMAA at physiological pH (7.00-7.40). Up to 86% of the 2-amino group is deprotonated at these pH values, resulting in a shared positive charge between the amino groups that is cancelled out by the net negative charge of the carboxyl group. Consequently, BMAA has no net charge at physiological pH levels. (Adapted from Nunn and Ponnusamy, 2009).

In a follow up study, Weiss *et al.* (1989) reported that bicarbonate significantly influences the ability of BMAA to activate NMDA receptors. This potentiating effect of bicarbonate was also observed for 2,4-DAB and 2,3-diaminopropanoic acid (DAP), amino acids that are not structurally characteristic of excitatory amino acids. These data indicated a mechanism by which a structurally 'non-excitatory' amino acid could induce excitotoxicity. Mroz (1989) showed that a BMAA carbamate could form via the rapid reversible covalent interaction between the unprotonated amino group of BMAA and carbon dioxide, which is present in equilibrium with bicarbonate. Under physiological conditions, the rate of carboxylation is determined by the concentration of the unprotonated amino group relative to the protonated form. Therefore an amino group with a relatively low pK_a will exist largely in an unprotonated form under physiological conditions, and consequently exist as a carbamate (Griffey *et al.*, 1988). Nunn and O'Brien (1989) investigated the interaction between BMAA and bicarbonate using ^{1}H-NMR and reported that the alpha amine group of L-BMAA reacts with a bicarbonate anion to form a α-*N*-carboxy BMAA adduct. Based on ^{1}H-NMR bicarbonate-BMAA interaction equilibrium data, Nunn and O'Brien (1989) stated that, in an aqueous medium, at physiological bicarbonate concentrations, up to 26% of L-BMAA could be

present as a different, modified species. Myers and Nelson (1990), using ^{13}C-NMR, investigated the covalent association between BMAA and bicarbonate and the associated carbamate/amine equilibrium. Under physiological conditions the majority of BMAA carbamate adducts were α-*N*-carboxy-BMAA, which was similar to the report by Nunn and O'Brien (1989) and in accordance with expected carboxylation of amino groups, as explained by Griffey *et al.* (1988). Moura (2009) confirmed the formation of BMAA carbamate adducts by NMR, and showed that *in vitro,* β-*N*-carboxy-BMAA can spontaneously and reversibly cyclise to form 1-methyl-2-oxoimidazolidine-4-carboxylic acid.

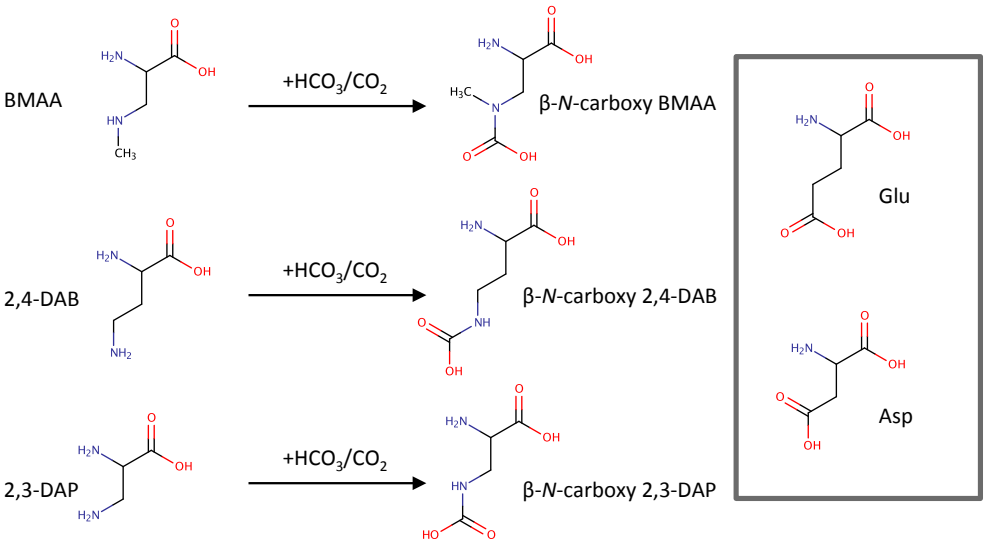

Figure 1.2: The reaction of diamino acids, BMAA, 2,4-DAB and 2,3-DAP, with bicarbonate at physiological pH to produce beta-carboxy adducts, as proposed by Weiss and Choi (1988). Weiss *et al.* (1989) suggested that the carboxy adducts of both BMAA and 2,4-DAB more closely resemble the chemical structures of excitatory amino acids, glutamate and aspartate (boxed).

Nunn *et al.* (1989) demonstrated that BMAA is a strong chelator of divalent metal ions zinc (Zn) and copper (Cu). L-BMAA forms complexes with Zn^{2+} and Cu^{2+} that are substantially more stable than corresponding glutamate complexes, and at pH 7.8 L-BMAA is highly likely to outcompete glutamate for binding with Zn^{2+} and even more so, with Cu^{2+}. Nunn *et al.* (1989) proposed that this strong chelation of Zn^{2+} and Cu^{2+} by BMAA could contribute to its acute neurotoxicity in that it binds to divalent metal ions in

the central nervous system (CNS), resulting in the disruption of metal ion metabolism and CNS dysfunction.

Other *in vitro* studies have shown that BMAA can be oxidised by L-amino acid oxidase and that the resulting iminium ion undergoes further chemical modification to yield a BMAA keto acid, and ultimately, *N*-methyl glycine (sarcosine) (Hashmi and Anders, 1991), and, that BMAA spontaneously reacts with pyridoxal-5'-phosphate to yield ammonia and methylamine (Nunn and Ponnusamy, 2009).

Biological role of BMAA

Cox *et al*. (2003) suggested, based on the toxicity of BMAA, that in cycads BMAA might function as a deterrent to herbivores. However, subsequent data showed that BMAA is produced by the symbiotic cyanobacteria within the cycad, making it more likely that BMAA is beneficial to the plant in some other way, such as nitrogen supply (Cox *et al*., 2003). Some have suggested, given that BMAA is a strong chaletor of metal ions (Nunn *et al.,* 1989) that the amino acid may function in iron binding in cyanobacteria (Bradley and Mash, 2009). At the end of 2010, nothing was known about why or when BMAA is produced, information that is important not only in understanding the environmental fate of BMAA, but also in predicting and reducing human exposure to this neurotoxin.

BMAA metabolism

Reece and Nunn (1988) reported on the metabolism of ^{13}C-labelled BMAA in the rat and showed that BMAA can be acetylated to form the BMAA derivative α-acetamido-β-methylaminopropionic acid. That this product was found in the urine, along with five other labelled compounds, strongly suggests that this identified compound was a detoxification product. Nunn and Ponnusamy (2009) demonstrated that in rat brain slices BMAA effects the levels of certain amino acids and results in an increase in ammonia, and that in rat liver or kidney homogenates the presence of BMAA results in changes in the levels of alanine, ammonia and/or methylamine and in the

appearance of the demethylated BMAA derivative, 2,3-DAP, the latter also presumably as a product of detoxification metabolism.

Biosynthesis of BMAA

In 2003 Brenner *et al.* proposed a two-step pathway for BMAA biosynthesis in cycads using expressed sequence tag analysis to identify putative genes that code for enzymes that may be involved in BMAA synthesis. A few years later, following the identification of putative orthologous genes of these enzymes in cyanobacterial genomes, Araoz *et al.* (2010) suggested that a BMAA biosynthetic pathway similar to that proposed by Brenner *et al.* (2003) could function in cyanobacteria. However, neither Brenner *et al.* (2003), nor Araoz *et al.* (2010) substantiated these proposed pathways with any biochemical data.

Within the field of BMAA research, studies on the metabolism, i.e. biosynthesis, catabolism and/or chemical modification of BMAA in any organism, have generally been very limited. By the end of 2010 there were very few data available on the fate of BMAA in any biological system. Understanding the metabolism of BMAA is central to understanding its mechanisms of toxicity, its persistence in the environment, and the extent of the risk of human exposure to this neurotoxin.

1.2.4 Environmental fate of β-*N*-methylamino-L-alanine

The potential for human exposure is a function of the environmental fate of BMAA, which in turn is determined by its chemical stability and potential for accumulation or metabolism by organisms in the environment.

Environmental persistence of BMAA

Bioaccumulation of a chemical is determined by various factors, including the chemical properties of the molecule, its concentration within the environment and the physiological properties of the organism that takes it up. Bioaccumulants tend to be compounds that are chemically stable and lipophilic and thereby likely to accumulate within fats, or compounds that have a high affinity for binding to proteins, thereby enabling and prolonging storage within an organism (Landrum and Fisher, 1998). BMAA, although not lipophilic, could be considered a potential bioaccumulant as it is chemically reactive at physiological pH and potentially has a high affinity for protein binding, as evidenced by the high concentrations of BMAA present in protein hydrolysates. Although the exact mechanism of bioaccumulation was not discussed, the first data on the environmental persistence of BMAA within an ecosystem were reported by Cox *et al.* (2003), showing that BMAA bioaccumulates and biomagnifies as a free amino acid throughout a food wed of the Guam ecosystem. This was expanded by Murch *et al*. (2004b) who showed that bioaccumulation and subsequent biomagnification of the neurotoxin throughout the same food web in the protein-associated form was substantially greater than for the free amino acid. Murch *et al.* (2004b) hypothesised that the mechanism of BMAA bioaccumulation is protein-BMAA association and possibly misincorporation of BMAA into protein. Arthur E. Bell (cited in Polsky *et al.,* 1972) commented that the association of BMAA with proteins is not necessarily at a primary level (misincorporation), but suggested that the protein-bound BMAA form is a low molecular weight compound, and hinted at an association between BMAA and a γ-glutamyl moiety.

BMAA, although chemically stable, is also chemically reactive (Nunn and Ponnusamy, 2009), as evidenced by the spontaneous reaction with bicarbonate (Weiss and Choi, 1988; Weiss *et al.,* 1989; Nunn and O'Brien, 1989; Mroz 1989; Myers and Nelson, 1990;), with the enzyme co-factor pyridoxal-5'-phosphate (Nunn and Ponnusamy, 2009), and with L-amino acid oxidase (Hashmi and Anders, 2001).

Theoretically, the relatively low pK_a of the 2-amino group of BMAA (Vega and Bell, 1967; Nunn and O'Brien, 1989) causes this group to be uncharged and therefore reactive at physiological pH (Nunn and Ponnusamy, 2009). The chemical reactivity of BMAA is an important factor to consider when evaluating its potential for bioaccumulation and its environmental fate. BMAA could readily react with cellular constituents and bioaccumulate within organisms, or BMAA could be readily metabolised within the environment. Either catabolism or chemical modification may yield metabolites with either reduced or enhanced toxicity. More importantly, metabolism of BMAA that involves the reversible covalent modification of the amino acid greatly impacts on the assessment of environmental BMAA concentrations and the perceived risk for human exposure, as BMAA may be present within the environment in a non-BMAA form that could readily convert to neurotoxic BMAA under specific conditions. Therefore, an understanding of the environmental fate of BMAA is necessary to clarify the large variations in reported environmental BMAA levels.

Potential for human exposure to neurotoxic BMAA

Concerns over the extent of human BMAA exposure heightened following the link between BMAA and cyanobacteria, and the need to identify potential routes of BMAA exposure was recognised.

Considering the environmental and global ubiquity of cyanobacteria, there are numerous potential routes of BMAA exposure, either via direct or indirect contact with these microorganisms. BMAA has been detected in cyanobacteria isolated from various freshwater lakes around the world, suggesting that there is potentially a high risk of human exposure to BMAA via direct or indirect contact with water from impoundments that experience recurrent cyanobacterial blooms. Metcalf and Codd (2009) highlighted various possible routes of human exposure to this neurotoxin based on the presence of BMAA in freshwater, however, the same risks could apply in marine environments. The direct ingestion or inhalation of cyanobacterial cells, during recreational activities or by drinking contaminated water, could potentially result in the bioaccumulation of BMAA within humans or animals.

Caller *et al*. (2009) suggested that a possible route of exposure to BMAA was via inhalation of aerosolised water droplets containing BMAA or cyanobacterial cells. This speculation arose following the identification of an ALS cluster (10-25 fold higher incidence than the expected average) situated around a lake that has frequent cyanobacterial blooms. Furthermore, water contaminated with cyanobacteria may be used for irrigation of crop plants, or drinking water for livestock, which may potentially lead to the bioaccumulation of BMAA within the exposed animals or plants. The bioaccumulation of the cyanotoxin, microcystin (MC) by salad lettuce (*Lactuca sativa)* following irrigation with water containing cyanobacteria, had been reported (Codd *et al.,* 1999), as well as the accumulation of MC in the seedlings of various agriculturally important crop plants (Peuthert *et al.,* 2007). However, by the end of 2010 the majority of data on the bioaccumulation of BMAA in plants, other than cycads, were from laboratory exposures, and were therefore only indicative of the potential for BMAA bioaccumulation in plants, rather than the actual presence of BMAA in plants within the environment. Laboratory scale studies have shown that BMAA is readily taken up by a variety of plants (Esterhuizen *et al.,* 2011; Contardo-Jara *et al*., 2013), including *Triticum aestivum* (Contardo-Jara *et al.,* 2014) and *Nasturtium officinale,* and *Daucus carota* (Niyonzima, 2011), but the metabolism and fate of BMAA in these plants were not investigated. Esterhuizen-Londt (2010) reported that no BMAA was present in selected crop plants irrigated with water containing cyanobacterial bloom material, despite the bloom material containing BMAA. Given the evidence of BMAA uptake by various plant species, the absence of BMAA in these crop plants does not necessarily mean that BMAA was not taken up, but could suggest that BMAA was metabolised within the plants.

The bioaccumulation of BMAA in animals has been well documented. A wide concentration range of BMAA, both in free and protein-associated forms has been detected in various aquatic invertebrates and vertebrates (Brand*,* 2009; Brand *et al.*, 2010; Jonasson *et al*., 2010; Mondo *et al.,* 2012), including species frequently consumed by humans such as mussels, oysters, crab, herring, and sharks (fins). There is also evidence for the potential bioaccumulation of BMAA in milk, as demonstrated by Andersson *et al.* (2013).

Following intravenous administration of BMAA, high levels of the amino acid was excreted in milk by nursing mouse dams, and BMAA was transferred via the milk to suckling mouse pups raising concerns over the potential for BMAA exposure to nursing human infants, and to all humans via the consumption of dairy products from other animals that had been exposed to BMAA via drinking water or feedstuff.

Other routes of exposure to BMAA have been suggested, and include the direct consumption of cyanobacteria, as practised in Peru (Johnson *et al*., 2008) and China (Roney *et al.,* 2009), the inhalation of aerosolised cyanobacterial desert crusts (Cox *et al.*, 2009) and the consumption of cyanobacterial dietary supplements (Dietrich *et al*., 2008).

Based on the large variations in the concentrations of BMAA detected in different animals and in different species of the same animal, Brand *et al.* (2010) suggested that the ability of animals, including humans, to bioaccumulate BMAA may vary. Factors such as the duration of BMAA exposure, the age of the animal at the time of exposure and differences in BMAA uptake and excretory mechanisms may play a role in determining the extent of BMAA bioaccumulation in a given animal. Therefore, understanding the metabolism of BMAA in environmentally relevant organisms is key to accurately gauge the potential for human exposure to this neurotoxin.

1.3 RESEARCH RATIONALE

The origin and biological role of BMAA remains uncertain and this uncertainty has brought into question not only the accuracy of BMAA analytical methods, but also the reported concentrations of BMAA within the environment and the associated risk of human exposure to this neurotoxin, and consequently the link between BMAA and neurodegenerative disease. Furthermore, nothing is known about how or when BMAA is made, knowledge that is important in understanding the reported variation in environmental BMAA concentrations. In addition, although evidence confirms the presence of BMAA within the environment, there are very limited data on the metabolism of BMAA by other organisms, and, the environmental fate of this neurotoxin, a key aspect in gauging the human exposure risk, is still unknown.

The aim of the research presented here was to gain a greater understanding of the origin and fate of BMAA, and in doing so address uncertainties that have been impeding the progression of research into the link between BMAA and the development of neurodegenerative disease. The objectives of the current study were therefore as follows:

1. To select a sensitive, reproducible and accurate analytical method for the detection and quantification of BMAA, in both free and protein-associated forms, within complex biological matrices.
2. To demonstrate with solid evidence that the neurotoxin, BMAA, is produced by cyanobacteria, by showing the production of labelled BMAA from a labelled starting substrate.
3. To investigate the metabolism, biosynthesis and biological role of BMAA in cyanobacteria.
4. To investigate the environmental fate of BMAA by examining the metabolism of BMAA in models representing environmentally relevant organisms.

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CHAPTER 2: SELECTING A SENSITIVE, REPRODUCIBLE AND ACCURATE ANALYTICAL METHOD FOR THE DETECTION AND QUANTIFICATION OF β-*N*-METHYLAMINO-L-ALANINE (BMAA) IN COMPLEX BIOLOGICAL MATRICES

2.1 INTRODUCTION

Since the discovery of BMAA in cycad seeds (Vega and Bell, 1967), it has been hypothesised that this neurotoxic amino acid may be implicated in the development of latent onset neurodegenerative motor neuron disease (Spencer *et al*., 1986, 1987; Nunn *et al.,* 1987; Ross and Spencer, 1987). However, this hypothesis soon received widespread criticism as it was argued that BMAA induced acute and not latent neurotoxicity in animal models and that BMAA doses used in these studies were unrealistic (Garruto *et al.,* 1988; Duncan *et al.,* 1988). Later investigations revealed that in biological tissues BMAA is present in a protein-associated form at much higher concentrations than those previously published for free amino acid fractions (Murch *et al*., 2004b) and that BMAA is not only present in cycads but also in diverse genera of free living cyanobacteria (Cox *et al.*, 2005). Following the first report of BMAA in cyanobacteria (Cox *et al.,* 2003), many more followed (Banack *et al*., 2007; Esterhuizen and Downing, 2008; Metcalf *et al.,* 2008; Johnson *et al.*, 2008; Lürling *et al.*, 2008). However, as different investigators employed different analytical methods in their attempts to analyse BMAA in cyanobacteria and other organisms, often using cycad material as a positive control, the presence of BMAA in cyanobacteria and animal tissue was not always confirmed (Rosén and Hellenäs, 2008; Krüger *et al.,* 2010; Li *et al*., 2010). Based on these discrepancies, controversy soon arose over the cyanobacterial origin of BMAA, the accuracy of analytical methods and the validity of reported BMAA cocentrations.

This chapter is based on three co-authored papers (provided in Appendix A); (1) Banack, S.A., Downing, T.G., Spáčil, Z., Purdie, E.L., Metcalf, J.S., **Downing, S**., Esterhuizen, M., Codd, G.A. and P.A. Cox. 2010. Distinguishing the cyanobacterial neurotoxin β-*N*-methylamino-L-alanine (BMAA) from its structural isomer 2,4-diaminobutyric acid (2,4-DAB). *Toxicon* 56(6): 868-79; (2) Banack, S.A., Metcalf, J.S., Spáčil, Z., Downing, T.G., **Downing, S.**, Long, A., Nunn, P.B. and P.A. Cox. 2011. Distinguishing the cyanobacterial neurotoxin β-*N*-methylamino-L-alanine (BMAA) from other diamino acids. *Toxicon.* **57:** 730-738; (3) Esterhuizen-Londt, M., **Downing, S.** and T.G. Downing. 2011. Improved sensitivity using liquid chromatography mass spectrometry (LC-MS) for detection of propyl chloroformate derivatised β-*N*-methylamino-L-alanine (BMAA) in cyanobacteria. *Water SA.* 37(2): 133-138.

Many different methods have been employed to analyse BMAA, including electrophoresis (Vega and Bell, 1967; Baptista *et al.,* 2011), ^{1}H-NMR (Moura, 2009) and automated amino acid analysers (Banack *et al.*, 2007). However, BMAA analytical methods using LC or GC coupled to optical or mass spectrometric detection have received the most consistent criticism (Rosén and Hellenäs, 2008; Krüger *et al.,* 2010). Of all the reported methods, ^{1}H-NMR spectroscopy, and LC coupled to triple quadrupole mass spectrometric detection, are the two most selective methods. Despite high selectivity, ^{1}H-NMR is not very sensitive (5mg/mL LOD reported by Moura *et al*., 2009) and requires specialised equipment that is not always readily accessible. Selection of a method for BMAA analysis was therefore narrowed to LC-based methods.

2.2 ANALYSIS OF BMAA IN COMPLEX BIOLOGICAL MATRICES USING LC-BASED METHODS

LC-based methods routinely employed in BMAA analysis can be divided into two groups; methods that include pre-column derivatisation and those that do not. Generally, derivatisation is used to improve analytical capabilities by altering the chemical or physical properties of a molecule to achieve enhanced separation, sensitivity and selection. When analysing low molecular mass analytes such as amino acids in a complex matrix like protein hydrolysates, derivitisation is often employed in order to shift the analyte signal to a mass range with lower chemical noise and less ion suppression and thereby increase analytical sensitivity. One concern expressed by some investigators that were unable to detect underivatised BMAA using HILIC-MS (Rosén and Hellenäs, 2008; Krüger *et al.,* 2010), was the possibility that following derivitisation other molecules or isomers of BMAA may have the same mass to charge ratio (*m/z*), resulting in misidentification of BMAA. However, misidentifying BMAA isomers or another compound as BMAA based on identical *m/z* is not unique to methods employing derivatisation, but also possible in underivatised samples.

This chapter is based on three co-authored papers (provided in Appendix A); (1) Banack, S.A., Downing, T.G., Spáčil, Z., Purdie, E.L., Metcalf, J.S., **Downing, S**., Esterhuizen, M., Codd, G.A. and P.A. Cox. 2010. Distinguishing the cyanobacterial neurotoxin β-*N*-methylamino-L-alanine (BMAA) from its structural isomer 2,4-diaminobutyric acid (2,4-DAB). *Toxicon* 56(6): 868-79; (2) Banack, S.A., Metcalf, J.S., Spáčil, Z., Downing, T.G., **Downing, S.**, Long, A., Nunn, P.B. and P.A. Cox. 2011. Distinguishing the cyanobacterial neurotoxin β-*N*-methylamino-L-alanine (BMAA) from other diamino acids. *Toxicon.* **57:** 730-738; (3) Esterhuizen-Londt, M., **Downing, S.** and T.G. Downing. 2011. Improved sensitivity using liquid chromatography mass spectrometry (LC-MS) for detection of propyl chloroformate derivatised β-*N*-methylamino-L-alanine (BMAA) in cyanobacteria. *Water SA.* 37(2): 133-138.

To avoid misidentification of different analytes with the same *m/z*, good chromatographic separation must be achieved and/or collision-induced dissociation of parent ions must produce unique product ions.

In selecting a method for BMAA analysis for use throughout the current study the following factors were considered: Reproducibility, ease of use and equipment availability, sensitivity, and precision and accuracy in complex biological matrices.

2.2.1 Analysis of underivatised BMAA using HILIC

HILIC, an alternative mode of high performance liquid chromatography first described in 1990 (Buszewski and Noga, 2012), is a relatively new method of amino acid analysis compared to the more traditional methods using ion exchange chromatography. It is commonly used to separate small polar compounds in complex systems, and is ideally suited to the separation of uncharged hydrophilic (or amphiphillic) molecules that are too polar for effective separation with RPLC, but that are too weakly charged for effective separation with ion exchange chromatography (Buszewski and Noga, 2012). HILIC separation is based on differences in analyte polarity and is achieved by interaction of polar molecules with a polar or hydrophilic stationary phase, followed by elution with increasing polar content of the mobile phase. HILIC separation can be coupled with different types of detectors, including UV and MS, however, in methods employing HILIC for separation of BMAA, detection has routinely been by single or triple quadruple MS (Rosén and Hellenäs, 2008; Kubo *et al.,* 2008 Krüger *et al.,* 2010; Li *et al.,* 2010; Faassen *et al.,* 2009).

HILIC-MS has been used for analysis of BMAA in different biological samples, including cycad seed extracts, cyanobacteria and brain tissue. Generally, this method has been successful in detecting BMAA in cycad extracts, but not in cyanobacteria or other

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biological matrices. Of the many independent studies in which HILIC-MS was used (Rosén and Hellenäs, 2008; Kubo *et al.,* 2008; Faassen *et al.,* 2009; Krüger *et al.,* 2010; Li *et al.,* 2010), only a single report of the presence of BMAA in cyanobacterial extracts was published (Faassen *et al.,* 2009) although the amino acid could be detected in spiked cyanobacterial extracts and in cycad seed extracts, and in some cases the BMAA isomer, 2,4-DAB, was detected in BMAA-negative samples. This cast doubt on the sensitivity of HILIC methods in detecting BMAA in complex matrices such as protein hydrolysates or in samples where the concentration of BMAA is potentially low (such as in cyanobacteria and brain tissue). Reported LOD's of HILIC methods used in BMAA analysis range from 0.017 to 4.0 pmol/injection (Li *et al*., 2010 and Kubo *et al*., 2008, respectively) and at 0.017 pmol/injection is comparable to the LOD of RPLC-MS methods that employ pre-column derivatisation (Banack *et al.,* 2010). That said, in most cases LOD's were determined using only amino acid standards and were not determined in the presence of a complex biological matrix. Reported LOD's are therefore not truly indicative of the method's sensitivity in complex matrices. This error is not unique to HILIC methods but is also present in most other reported LC-MS methods used for BMAA anlaysis, where instrument or method LOD's are reported rather than practical LOD's.

HILIC-MS, like other LC-MS methods identifies analytes based on four selection criteria; retention time, *m/z* of parent ions, *m/z* of product ions produced during collision-induced dissociation of parent ions, and the ratios of product ions (Buszewski and Noga, 2012). However, although HILIC-MS is theoretically suited to the analysis of BMAA, sensitivity, reproducibility and accuracy of BMAA quantification in complex biological matrices using HILIC-MS has not been practically demonstrated (Rosén and Hellenäs, 2008; Kubo *et al.,* 2008; Faassen *et al.,* 2009; Krüger *et al.,* 2010; Li *et al.,* 2010). For this reason, HILIC-MS was not considered as a suitable method for the quantification of BMAA in complex biological matrices.

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2.2.2 Analysis of derivatised BMAA

LC with optical detection - RPLC-UV and RPLC-FD

Mass analysers, as used in LC-MS methods, are capable of discriminating between different analytes based on parent or product ion *m/z* thereby increasing analytical sensitivity and accuracy. However, optical detectors are non-discriminating and this means that there is a relatively high possibility of analyte misidentification in complex matrices, especially when analysing low molecular mass molecules. Analytical sensitivity and accuracy of LC methods using optical detection rely largely on good chromatographic separation and this often entails long run times.

The first report of derivatisation of BMAA using a fluorogenic derivatising agent was in 1988 (Kisby *et al*.). BMAA was derivatised with FMOC and separated by gradient elution over 30 minutes. The reported LOD was 0.6 pmol/injection and the method was successful in detecting BMAA in cycad extracts, serum and CSF. However, the sensitivity of this method in a biological matrix could not be reproduced by Montine *et al.* (2005), who modified the 30 minute combined linear and isocratic gradient of Kisby *et al.* (1988) to a purely linear gradient over 63 minutes, and could not detect BMAA in brain tissue.

The most common derivatising agent used for BMAA analysis with optical detection is AQC (Waters AccQ-Tag™). Various studies using HPLC-FD or UPLC-UV of AQC derivatised BMAA have reported the positive detection of BMAA in plant and animal tissue (Banack and Cox*,* 2003a,b), in brain tissue (Murch *et al.,* 2004a,b; Pablo *et al.,* 2009), and cyanobacteria (Cox *et al.,* 2003; Murch *et al.,* 2004b; Banack *et al.,* 2007; Metcalf *et al.,* 2008; Johnson *et al.,* 2008). However, adequate separation requires relatively long run times (39-60 mins as reviewed in Banack *et al.,* 2010) and there is no confirmation of analyte identity based on ion *m/z*. The separation efficiencies of these methods are compared in Table 2.1.

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The reported LOD for UPLC-UV is 0.1 pmol/injection (Banack *et al*., 2007) and the reported LOD's for HPLC-FD range from 65 pmol/injection (Cox *et al.,* 2005) to 12 nmol/injection (Eriksson *et al.,* 2009). In some cases where identical HPLC-FD methods were used on identical equipment, the reported LOD's varied substantially (130 pmol/injection reported by Banack and Cox, 2003b and 65 pmol/injection reported by Cox *et al.,* 2005). This may suggest errors in the quantification of the LOD or improvements in derivatisation efficiency and method sensitivity, but also raises concern over the reproducibility of this method.

Table 2.1: The efficiency of diamino acid separation of various BMAA analysis methods that employ derivatisation with either AQC or PCF. The total run time of each method is indicated in brackets. (*) Double chromatographic peaks produced by 2,6-diaminopimelic acid (DPM). BMAA isomers that have the same *m/z* as BMAA following derivatisation are highlighted. [1]Banack and Cox, 2003; [2,3]Banack *et al*., 2007; [4]Banack *et al*., 2010). Asn (asparagine), Glu (glutamate), 2,6 DPM (2,6-diaminopimelic acid), AEG (*N*-2(aminoethyl)glycine), 2,4 DAB (2,4-diaminobutyric acid), Orn (ornithine), Lys (lysine).

Diamino acid	Difference in retention time from that of BMAA (min)			
	AQC (Waters AccQ-Tag™)			PCF (EZ:faast™)
	HPLC-FD[1] (58 min)	HPLC-UV[2] (9.5 min)	HPLC-MS[3] (9.5 min)	HPLC-MS[4] (17 min)
Asn	17.56	2.9	2.89	3.96
Glu	16.06	2.45	2.45	3.48
2,6 DPM	10.33 and 8.13*	0.02 and 0.36*	0.02 and 0.34*	3.23
AEG	0.83	0	**0**	**0.35**
2,4 DAB	2.21	0.13	**0.12**	**1.27**
Orn	2.73	0.99	0.99	0.94
Lys	3.91	1.76	1.77	0.09

Interestingly, in almost all studies in which HPLC-FD/UV analysis of AQC derivatised BMAA was used, results were verified using either LC-MS or LC-MS/MS. This again highlights the fact that optical detection is not an accurate or reliable method of BMAA quantification in complex matrices.

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As the identity of the analyte cannot be confirmed, the potential for misidentification or over estimation of BMAA by these methods is high and therefore methods using optical detection were excluded in the selection of a suitable BMAA analysis method. Based on the evaluation of HILIC-MS, HPLC-FD and HPLC-UV methods in light of the set method selection criteria; reproducibility, ease of use and equipment availability, sensitivity, and precision and accuracy in complex biological matrices, it was clear that none of these methods adequately met these criteria and that for the purpose of this study LC-MS was deemed the best analytical platform for quantification of BMAA in biological matrices.

LC with mass spectrometric analysis of derivatised BMAA

There are two derivatising agents that have been used routinely in LC-MS analysis of BMAA; AQC and PCF, and each one is employed in unique analytical methods (Figure 2.1).

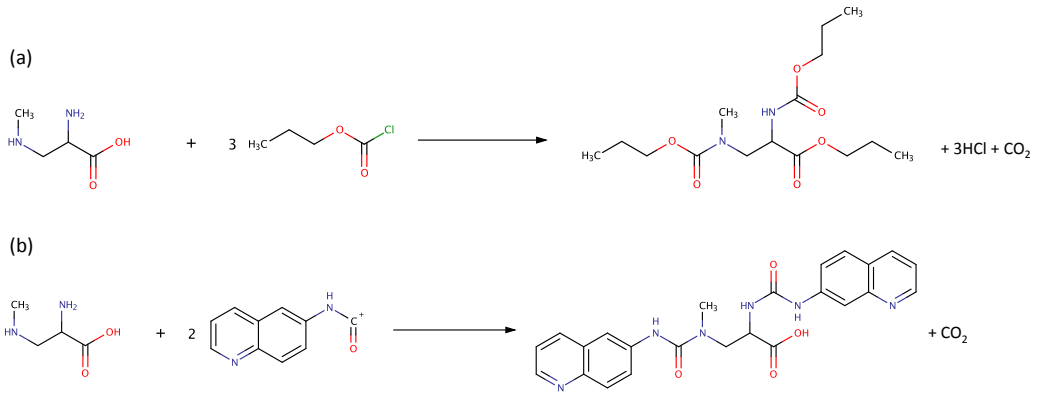

Figure 2.1: BMAA derivatisation reactions of (a) EZ:faast™ and (b) AccQ-Tag™.

Waters AccQ-Tag™

AQC is used as a commercially available derivatisation kit (Waters AccQ-Tag™), consisting of a borate buffer, dry derivatising reagent powder and reagent diluent. The protocol involves firstly, the reconstitution of the AccQ-Tag™ reagent powder in diluent,

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which is then added to the sample together with borate buffer. The sample is then mixed and incubated at room temperature for one minute followed by incubation at 55 °C for ten minutes, after which the sample is ready for analysis. The highly reactive AQC binds to both primary and secondary amines, yielding fluorescent derivatives (Figure 2.1). AccQ-Tag™ has been successfully used in the analysis of amino acids including BMAA and has the advantage that the derivatising technique is quick (<15 min) and easy and highly reproducible, and is linear over a broad range of hydrolysate amino acid concentrations (2.5-200 µM) (Cohen and Michaud, 1993). Furthermore, AQC derivatives are stable for up to one week and, the reagent, due to a short half-life, produces a minimum of interfering reactive products (Cohen and Michaud, 1993).

Although AccQ-Tag™ was designed to be used in HPLC or UPLC separation coupled with optical detection, many have combined the use of this kit with MS detection. However, while the AQC adducts and the eluents used in AccQ-Tag™ UPLC-UV/FD are compatible with MS analysis, Salazar *et al.* (2012) commented that the non-volatile borate buffer, needed to provide the optimum pH to obtain the highest derivatisation yield, may interfere with the optimisation of MS parameters, especially during direct infusion. This can be overcome by using a different buffer (Salazar *et al.,* 2012). Another shortfall of AccQ-Tag™ is the absence of specific guidelines to LC elution gradients. The original method as published by Cohen and Michaud (1993) reported complete resolution of all amino acids tested in less than 35 min. However, other methods using AccQ-Tag™ and LC-MS reported gradient elution of 10 min (Murch *et al*., 2004a) and 25 min (Cox *et al.,* 2005). Because the kit does not give chromatographic specifications such as gradient conditions, individual users modify gradient profiles in order to decrease analysis time, often at the expense of sensitivity and accuracy. AccQ-Tag™ also does not include an internal derivatisation standard that can be used to determined method precision, represented by a percentage relative standard deviation (%RSD) of the analyte to internal standard peak areas obtained from n>5 replicates. Some users have used internal standards such as nor-leucine (Cox *et al*., 2005) and DAVA (Kisby *et al.,* 1988),

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but this again is up to the individual user to include and is not standardised by the kit, affecting method reproducibility and precision. Eriksson *et al.* (2009) demonstrated how the concentration of protein in a sample could affect the derivatisation efficiency of AccQ-Tag™, the amount of derivatised BMAA produced and consequently the accuracy of BMAA quantification. The rate of reaction between AQC and different amino acids varies due to different binding affinities of primary and secondary amino groups and this may result in variation in derivatisation efficiency of specific analytes (Cohen and Michaud, 1993). If a sample contains molecules with a higher affinity for AQC compared to the analyte in question (BMAA), as is often the case in a complex biological matrix, molecules with a higher affinity will outcompete BMAA for binding to AQC resulting in the underestimation of BMAA concentrations. In order to avoid such variations in deriviatisation efficiency it is imperative that the AccQ-Tag™ is present in the correct concentration range relative to the analyte in question. Cohen and Michaud (1993) recommended a molar reagent excess of three or more in order to achieve an optimum derivatisation yield. Eriksson *et al.* (2009) showed that as the concentration of protein in the sample increases, the molar excess of AccQ-Tag™ decreases and consequently the peak area of BMAA decreases. In order to maintain quantification accuracy and precision, it is necessary to first determine the optimum sample concentration that would give an optimal derivatisation yield of the analyte in question. This step is often ignored in methods were AccQ-Tag™ is used, which raises concern over the accuracy and precision of the data obtained and the reproducibility of the method in complex biological matrices.

Phenomenex EZ:faast™

PCF derivatisation of BMAA for LC-MS analysis can be achieved using a commercial kit, Phenomenex EZ:faast™ Free (physiological) Amino Acid LC-MS. Although the kit specifies "free amino acids" it is also well suited for analysis of protein hydrolysates, given that

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the hydrolysates are dried down and reconstituted in 20mM HCl prior to derivatisation. The protocol involves the addition of an internal standard solution to the sample. The internal standard solution is buffered to ensure that the sample is at the correct pH for optimal solid phase extraction (SPE) efficiency. SPE of the sample is performed by passing it through a proprietary cation exchange resin. The internal standard buffered solution ensures that amino acids are in an ionic form and fit for cation binding. This SPE step ensures that interfering substances are removed, thereby reducing chemical noise and improving analytical sensitivity (Badawy, 2012). Following SPE, amino acids are released from the solid phase by addition of an alkaline elution medium. Amino acids are thereafter derivatised with PCF, forming derivatives at primary and secondary amine groups as well as carboxyl groups (Figure 2.1) (Badawy, 2012). Derivatised amino acids are then extracted from the aqueous phase into the organic layer following a phase separation step. The organic phase is dried off under a stable stream of nitrogen gas, and reconstituted in the LC mobile phase. This ensures a highly pure derivatised sample free of interfering compounds. Amino acid derivatives are stable at room temperature for only a few hours but are stable at 4 °C for a few days. LC-MS conditions and the elution gradient profile is outlined in the kit manual, with complete resolution of 50 amino acids in 17 minutes (12 minutes run time, 5 minutes re-equilibration). The kit manual also conveniently lists LC-MS parent ions and all possible LC-MS/MS product ions of 50 amino acids. This makes the use of EZ:faast™ ideally suited to studies in which other amino acids apart from BMAA are also of interest.

Evaluation of AccQ-Tag™ and EZ:faast™ methods based on published data

In theory, both AccQ-Tag™ and EZ:faast™ methods are suited to the quantification of amino acids including BMAA in complex biological matrices. However, in order to select the most suitable method for BMAA and general amino acid anlaysis, the actual

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performance of AccQ-Tag™ and EZ:faast™ methods as reported in published data were evaluated based on sensitivity, accuracy, precision, reproducibility and ease of use.

Sensitivity

The sensitivity of these methods, presented by reported LOD's is shown in Table 2.2. All the reported LOD's were determined using only amino acid standards, and not in the presence of a complex matrix. However, the LOD of the EZ:faast™ method was determined in the presence of 20 other amino acids (200 µM each), which gives a little more credence to this method's LOD. The reported LOD's for all AccQ-Tag™ based methods were with LC-MS detection using a triple quadrupole mass analyser. The EZ:faast™ LOD is for LC-MS with single quadrupole mass analyser, and the use of LC-MS/MS with single reaction moninotoring (SRM) further improves this LOD (Esterhuizen-Londt *et al*., 2011). Based on these data, EZ:faast™ LC-MS(MS) analysis is the most sensitive method for BMAA detection.

Table 2.2: Summary of method sensitivity based on the reported LOD's of methods using AccQ-Tag™ or EZ:faast™ derivatisation with LC-MS analysis. [1]Banack *et al*., 2010; [2]Spáčil *et al*, 2010; [3]Esterhuizen-Londt et al., *2011*. (*) Where all other LOD's were determined only in the presence of pure BMAA standard, this LOD was determined in a matrix of 20 other amino acids.

	AccQ-Tag™ without SPE	AccQ-Tag™ with SPE	EZ:faast™
LOD (pmol/injection)	0.070[1] – 0.170[1]	0.070[2]	0.042[3] *

Pre-derivatisation sample clean-up using SPE with AccQ-Tag™ LC-MS has been reported (Murch *et al.*, 2004a,b; Spáčil *et al.,* 2010). The value of including solid phase extraction of amino acid extracts prior to derivatisation and LC-MS, and the benefits it may have for analytical sensitivity and accuracy have been demonstrated by Spáčil *et al.,* 2010. The use of SPE substantially decreases the amount of interfering molecules without the loss

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of analytes and can enhance method sensitivity, which is clearly evidenced by the high sensitivity of the EZ:faast™ method.

Accuracy and Separation of BMAA from confounding isomers

A common concern in LC-MS analysis is the co-elution of molecules that share the same *m/z*. If this cannot be overcome by ensuring complete chromatographic resolution of analytes, the identity of analytes can be confirmed based on detection of unique product ions and the ratio of these ions, using triple quadrupole mass spectrometry. Reported biologicallly relevant isomers of BMAA are 2,4 DAB and AEG. However, when analysing complex biological matrices of which the composition is largely unknown, the potential for the presence of other molecules that have the same *m/z* as BMAA following derivatisation is very high. Therefore, even with complete chromatographic resolution of BMAA isomers it is necessary to confirm the identity of analytes using single or triple quadrupole LC-MS.

The chromatographic resolution of AccQ-Tag™ is not adequate to completely separate BMAA from AEG, and therefore, single quadrupole MS alone cannot be used to accurately quantify BMAA (Figure 2.2) and the use of LC-MS/MS based analysis of product ions is necessary. In contrast, the EZ:faast™ method completely separates BMAA from AEG and 2,4-DAB, and therefore BMAA can be accurately quantified using single quadrupole MS and the use of triple quadrupole MS is not necessary but only serves as confirmation.

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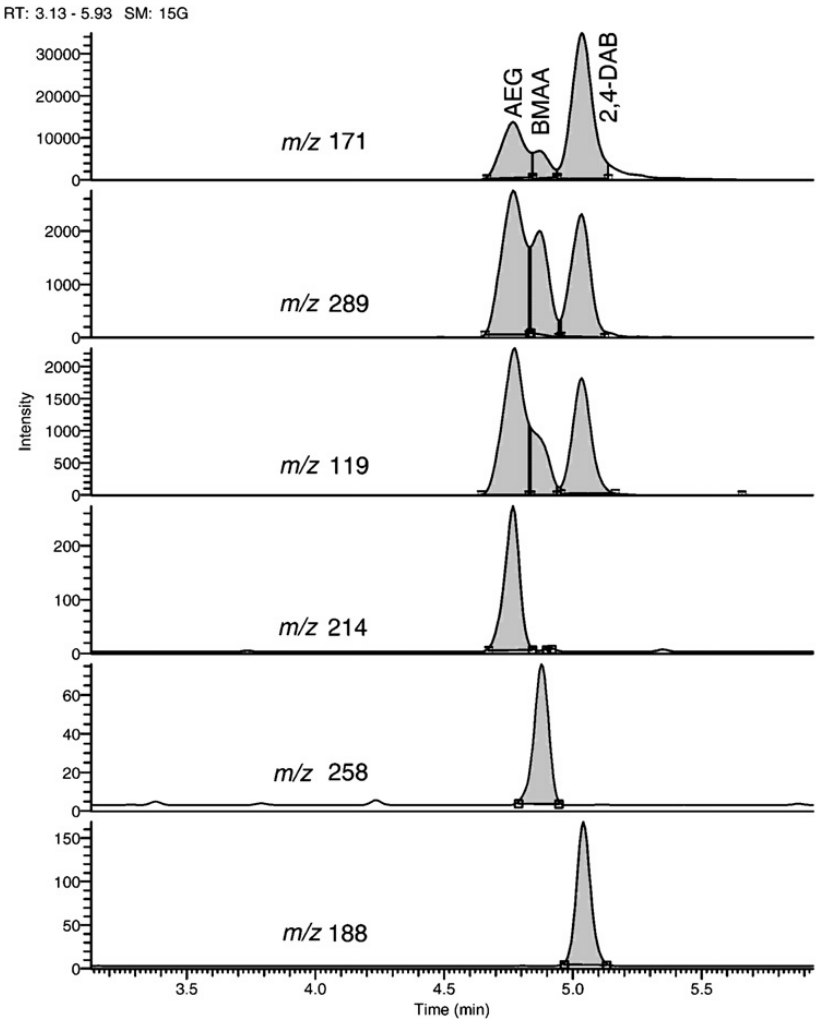

Figure 2.2: UHPLC-MS/MS of BMAA and BMAA isomers (2,4-DAB and AEG) in a mixture of 12 amino acids, derivatised with AccQ-Tag™. Collision-induced dissociation of parent ion (*m/z* 459) shared by BMAA, 2,4-DAB and AEG produces six product ions; *m/z* 171, 289, 119, 214, 258 and 188. Due to co-elution of parent ions, identification and quantification of AEG and BMAA relies solely on the detection of the unique product ions, *m/z* 214 and *m/z* 258, respectively. Adapted from Banack *et al.* (2011).

In a complex martrix, confirmation of analytes using triple quadrupole MS is always prudent. With AccQ-Tag™, collision-induced dissociation of parent ions of BMAA and its isomers (*m/z* 459) produces six product ions, three of which are common to all three isomers, and each isomer having one unique product ion (Figure 2.2). The presence of one unique product ion for each isomer partly overcomes their incomplete

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chromatographic separation. However, when this method is applied in a biological matrix, the unique product ions are not always detectable, in which case the ratios of non-unique product ions are sometimes used to confirm presence or absence of BMAA.

With EZ:faast™, there is complete separation of isomers (Table 2.1) and detection and quantification does not rely on MS/MS analysis. Collision-induced dissociation of parent ions of BMAA and its isomers derivatised with EZ:faast™ (*m/z* 333) produces three product ions, however, there are no product ions that are unique to either isomer, but unique product ion ratios can be used as additional confirmation of analyte identity (Figure 2.3). With this method, derivatisation of tryptophan also produces an *m/z* of 333, but collision-induced dissociation of this ion produces a product ion (*m/z* 230) that is unique to tryptophan.

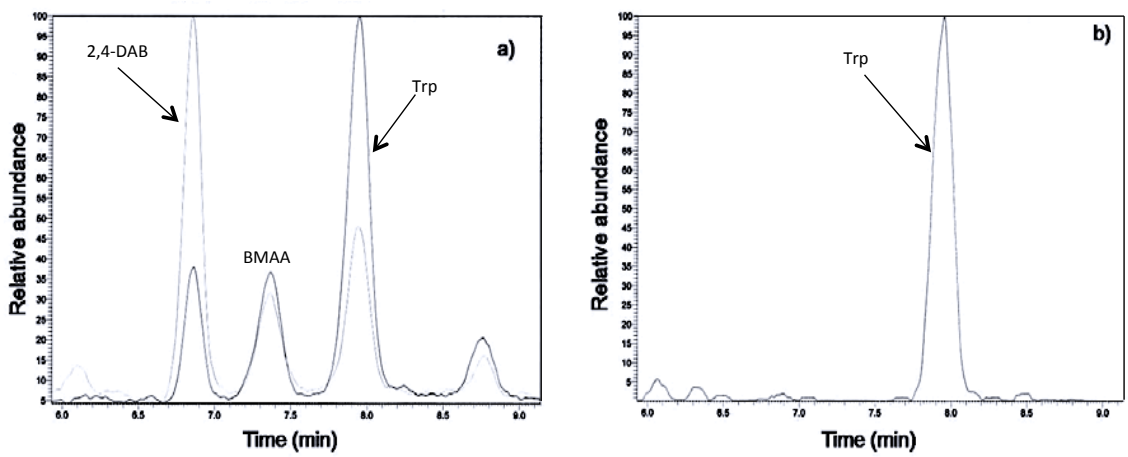

Figure 2.3: EZ:faast™ method: HPLC-MS/MS chromatogram of product ions produced by collision-induced dissociation of common parent ion *m/z* 333. (a) *m/z* 245 (dark line) and *m/z* 273 (light line) of BMAA and 2,4-DAB and tryptophan (trp) and (b) *m/z* 230, unique to trp. The absence of product ions that are unique to each amino acid is not critical as chromatographic separation is complete and analysis of product ions is purely for confirmation of analyte identity. Adapted from Esterhuizen-Londt *et al*. (2011).

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Based on the chromatographic separation of both methods, summarised in Table 2.1, it is clear that the EZ:faast™ method provides greater accuracy through complete chromatographic separation. It also provides greater ease of use, and the use of a triple quadrupole mass analyser is not necessary for BMAA detection or quantification.

Considering that BMAA can be present as BMAA-carboxy adducts at physiological pH (Mroz, 1988; Nunn and O'Brien, 1989) the underestimation of BMAA concentrations in free amino acid extracts may be possible. Cohen (2012) argued that the trichloroacetic acid (TCA) used in amino acid extractions provides a low pH to ensure the dissociation of BMAA-carbamate adducts. Although this is also valid for the EZ:faast™ method, the EZ:faast™ method derivatises both amine and carboxyl groups, resulting in the formation of derivatives of BMAA and BMAA-carboxy adducts, all of which have the same parent *m/z* of 333. This ensures that no BMAA present in a sample as a carbamate adduct is excluded from analysis, ensuring accuracy of detection and quantification of BMAA.

Precision

The precision of an LC-MS analytical method is determined as a %RSD of the analyte to internal standard peak areas and it gives an indication of the derivatisation efficiency, specifically in a complex biological matrix. As highlighted by Eriksson *et al.* (2009), when a sample is too concentrated, other amino acids may outcompete BMAA for binding with the derivatising agent, resulting in erroneous quantification of BMAA. To avoid this, the derivatising agent must be in molar excess of the anlaytes. This problem of inefficient or incompletely derivatisation can be overcome with the use of an internal standard. The EZ:faast™ method includes three internal standards that are added to the sample prior to SPE and derivatisation. These internal standards elute at different positions throughout the elution gradient, ensuring adequate representation of

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chemically diverse analytes in terms of SPE extraction recovery, chromatographic separation and ionisation response. Furthermore, although optimization of sample concentration is still necessary to ensure efficient derivatisation, the capacity of the SPE corresponds with the concentration of the derivatising agent added, ensuring optimum yield of amino acid derivatives. The AccQ-Tag™ kit does not include the use of an internal standard. Although individual users can add internal standards, the fact that it is not standardised by the kit means the use may be variable, ultimately compromising the reproducibility of the method and method precision.

Reproducibility

The EZ:faast™ method is available as a commercial standardised kit. This means, in contrast with the AccQ-Tag™ method, that the LC column, the elution gradient, the internal standards and SPE are all standardised and can be exactly replicated by anyone. Considering the AccQ-Tag™ method, it is difficult to guarantee reproducibility when so many aspects of the method are at the discretion of the individual user. Overall, both methods are easy to use, and perhaps at first glance sample preparation with the AccQ-Tag™ method appears to be faster. However, considering that the added steps in EZ:faast™ sample preparation, such as SPE, should also be included in the AccQ-Tag™ method to ensure optimum sensitivity and accuracy, the all-inclusive nature of the EZ:faast™ method makes it not only faster but far more reproducible. Results obtained using EZ:faast™ on several instruments have been directly comparable. This absence of intermachine/interlaboratory variation in analysis of a particular sample is also highly advantageous.

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2.3 CONCLUSION

Methods that use LC-MS detection of derivatised amino acids are more suitable to quantification of BMAA in complex biological matrices, and based on the above evaluation of commonly used BMAA analysis methods, the use of HILIC, HPLC-UV/FD is not recommended.

Both methods that employ pre-column derivatisation of amino acids, EZ:faast™ and AccQ-Tag™, proved suitable for BMAA analysis in complex matrices. However, from the evaluation of published data, and personal use of both systems, it was clear that the EZ:faast™ method is superior to the AccQ-Tag™ method in terms of sensitivity, accuracy, precision, reproducibility and ease of use, and was therefore used in the quantification of BMAA and analysis of other amino acids in all experiments that investigated the metabolism and environmental fate of BMAA.

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This chapter is based on three co-authored papers (provided in Appendix A); (1) Banack, S.A., Downing, T.G., Spáčil, Z., Purdie, E.L., Metcalf, J.S., **Downing, S**., Esterhuizen, M., Codd, G.A. and P.A. Cox. 2010. Distinguishing the cyanobacterial neurotoxin β-*N*-methylamino-L-alanine (BMAA) from its structural isomer 2,4-diaminobutyric acid (2,4-DAB). *Toxicon* 56(6): 868-79; (2) Banack, S.A., Metcalf, J.S., Spáčil, Z., Downing, T.G., **Downing, S.**, Long, A., Nunn, P.B. and P.A. Cox. 2011. Distinguishing the cyanobacterial neurotoxin β-*N*-methylamino-L-alanine (BMAA) from other diamino acids. *Toxicon.* **57:** 730-738; (3) Esterhuizen-Londt, M., **Downing, S.** and T.G. Downing. 2011. Improved sensitivity using liquid chromatography mass spectrometry (LC-MS) for detection of propyl chloroformate derivatised β-*N*-methylamino-L-alanine (BMAA) in cyanobacteria. *Water SA.* 37(2): 133-138.

Having selected the most sensitive, accurate and reproducible method for BMAA analysis, the controversial question of the biological origin of BMAA could now be addressed.

Although BMAA had been detected in cyanobacteria in many independent studies using different analytical methods, the cyanobacterial origin of BMAA was still disputed and unverified. Therefore, that BMAA is a cyanobacterial metabolite, had to be proven irrefutably.

CHAPTER 3: NITROGEN STARVATION OF CYANOBACTERIA RESULTS IN THE PRODUCTION OF β-*N*-METHYLAMINO-L-ALANINE

ABSTRACT

β-*N*-methylamino-L-alanine, an unusual amino acid implicated in neurodegenerative disease, has been detected in cultures of nearly all genera of environmentally ubiquitous cyanobacteria tested. The compound is present within cyanobacterial cells in free and protein-associated forms, with large variations occurring in the concentration of these pools between species as well as within single strains. With a lack of knowledge and supporting data on the regulation of BMAA production and the role of this compound in cyanobacteria, the association between BMAA and cyanobacteria is still subject to debate. In this study we investigated the biosynthesis of BMAA in axenic non-diazotrophic cyanobacterial cultures using the stable isotope ^{15}N. Nitrogen starvation of nutritionally replete cells resulted in an increase in free cellular ^{15}N BMAA suggesting that BMAA may be the result of catabolism to provide nitrogen or that BMAA is synthesised to serve a functional role in the cell in response to nitrogen deprivation. The addition of NO_3^- and NH_4^+ to the culture medium following starvation resulted in a decrease of free cellular BMAA without a corresponding increase in the protein-associated fraction. The use of ammonia as a nitrogen source resulted in a more rapid reduction of BMAA when compared to nitrate. This study provides the first data regarding the regulation of intracellular BMAA concentrations in cyanobacteria with results conclusively showing the production of ^{15}N BMAA by an axenic cyanobacterial culture.

Keywords: metabolism, ecophysiology, toxin, cyanobacteria

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3.1 INTRODUCTION

β-*N*-methylamino-L-alanine (BMAA), an unusual amino acid first isolated from the seeds of *Cycas micronesica* Hill [Cycadaceae] (Vega and Bell, 1967), elicits neurotoxicity via various mechanisms (Rao *et al.*, 2006; Lobner *et al.*, 2007; Liu *et al.*, 2009) and is a putative environmental trigger for Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex (ALS/PDC) as well as Amyotrophic Lateral Sclerosis (ALS) and possibly Alzheimer's disease (AD) (Murch *et al*., 2004; Pablo *et al*., 2009). BMAA is produced by diverse taxa of cyanobacteria (Cox *et al.*, 2005), in both free and protein-bound forms. BMAA was detected in 95% of all cyanobacterial genera and 97% of all cyanobacterial strains tested, which included brackish water, freshwater and marine isolates, as well as both free-living and symbiotic strains. Subsequently, various independent researchers have detected the neurotoxin in cyanobacteria isolated from marine, terrestrial and/or freshwater environments, using an array of detection methods (Banack *et al.,* 2007; Esterhuizen and Downing, 2008; Esterhuizen *et al.,* 2011a; Metcalf *et al.*, 2008; Bidigare *et al.*, 2009; Faassen *et al.*, 2009; Spáčil *et al.*, 2010). However, other investigators have been unable to detect BMAA in other laboratory cultured cyanobacterial strains (Rosén and Hellenäs, 2008; Krüger *et al.*, 2010) resulting in the association between BMAA and cyanobacteria being challenged. The ongoing controversy surrounding the link between BMAA and cyanobacteria relates to the possible misidentification of the compound during analysis. However, robust method development has produced methods that clearly and distinctly distinguish BMAA from known isomers as well as other diamino acids (Banack *et al.*, 2010; 2011).

Ongoing research continues to substantiate the neurotoxicity of BMAA in mammals while also contributing to knowledge surrounding the effect of the compound in invertebrates and plants (Brenner *et al.*, 2000; Purdie *et al.*, 2009; Esterhuizen *et al.*, 2011b, Esterhuizen *et al.*, 2011c). However, to date there are no published data on the biochemical role of BMAA in cyanobacteria or the biosynthesis of the compound by cyanobacteria. Brenner *et al.* (2003), having identified expressed gene sequences encoding enzymes that might support a hypothesised pathway, proposed a simple

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two-step biosynthetic pathway for BMAA in *Cycas* spp. in which cysteine, phosphoserine, o-acetylserine or cyanoalanine were possible starting points for BMAA biosynthesis. In his proposed pathway ammonia is transferred to the β-carbon of a β-substituted alanine (cysteine, phosphoserine, o-acetylserine or cyanoalanine), a reaction catalysed by a cysteine synthase-like enzyme. This is followed by methylation of the reaction intermediate with *S*-adenosylmethionine acting as the methyl group donor. Although possible genes encoding both cysteine synthase-like and methyltransferase enzymes were identified within the cycad expressed sequence gene tag library, the existence of such enzymes with specificity for the proposed substrates of this pathway remains speculative. Data on BMAA biosynthetic pathways in cyanobacteria are currently lacking, although Araoz *et al.* (2010) recently suggested that the cycad BMAA biosynthetic pathway proposed by Brenner *et al.* (2003) could also occur in cyanobacteria.

Although improved analytical methods for the detection of BMAA in a complex matrix have substantiated the link between this neurotoxin and cyanobacteria, evidence of its production from supplied raw materials by cyanobacteria is still lacking. We therefore sought to confirm the biosynthesis of BMAA in an axenic cyanobacterial culture based on the incorporation of the stable isotope ^{15}N and the subsequent detection of ^{15}N labeled BMAA in cellular extracts. Additionally, the use of this isotope allowed for the determination of the time frame of BMAA production in comparison to that of *de novo* synthesised proteinogenic amino acids.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Strains and culture conditions

An axenic *Microcystis* PCC7806 culture was obtained from the Pasteur Culture Collection (Paris, France) and a uni-algal strain, *Synechocystis* J341, was obtained from the Cyanobacterial Research Group, Nelson Mandela Metropolitan University, South Africa (referred to from here on as *Microcystis* and *Synechocystis,* respectively). Cultures were

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maintained in BG_{11} media (Rippka, 1988) under sterile conditions at a temperature of 23 °C (±1°C) with constant illumination at a light intensity of 16 µmol m^{-2} sec^{-1} (Triton Dayglo©, Cleveland, Ohio). Culture purity was regularly monitored microscopically and by heterotrophic culture.

3.2.2 Short term nitrogen starvation followed by short term ^{15}N exposure

In order to determine whether BMAA is synthesised by transamination reactions during the synthesis of proteinogenic amino acids, short term ^{15}N exposures of the cultures were performed. Nutritionally replete mid-log *Microcystis* culture replicates were nitrogen starved for 24 hours in order to ensure nitrogen uptake on exposure to ^{15}N; cells were collected via centrifugation (5000 × g for 10 minutes) and resuspended to an 0.6_{OD} at 740_{nm} in nitrogen-free BG_{11}^{0} media (Rippka, 1988). Following nitrogen deprivation, experimental culture replicates were supplemented with 1mM ^{15}N (NH_4Cl) and control cultures with 1 mM ^{14}N ($NaNO_3$). Cells were harvested from 10 mL samples by centrifugation (5000 × g for 20 min) at 0 min, 10 min, 1 h, 4 h and 24 h.

In order to analyse the distribution of labelled amino acids, including BMAA, that were released from proteins or intracellular nitrogen stores, or synthesised during nitrogen starvation, the nutritionally replete cultures that had been nitrogen deprived for 24 h prior to nitrogen supplementation for 24 h, were nitrogen starved again for 24 h, harvested by centrifugation and all cell pellets stored at -20 °C pending extraction. All culture treatments were replicated three times.

3.2.3 Long term ^{15}N exposure followed by long term nitrogen deprivation

Based on the data obtained from short term ^{15}N exposures, the experiments were repeated allowing a longer ^{15}N exposure time. Nutritionally replete mid-log *Microcystis* culture replicates were deprived of nitrogen for 24 h prior to supplementation of experimental and control cultures with ^{15}N and ^{14}N, respectively, as described before.

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Cultures were grown, cells were collected by centrifugation at 90 days followed by nitrogen deprivation through growth in nitrogen-free BG_{11}^{0} media as before. Samples were taken at 24, 48 and 192 h.

In order to confirm data obtained during long term exposure of *Microcystis* to ^{15}N, a second long term exposure experiment was performed with unicellular non-nitrogen fixing *Synechocystis*. Culture replicates were nitrogen deprived for 24 h prior to medium supplementation with ^{14}N and ^{15}N as described before. Cultures were sampled (as described above) at 10 days following supplementation and nitrogen deprived in BG_{11}^{0} over 120 h with samples taken periodically. All treatments were replicated three times and cell sample pellets were stored at -20°C pending extraction.

3.2.4 Extraction and analysis of ^{15}N-labelled amino acids

Free and protein-associated amino acids were extracted from cell pellets using trichloroacetic acid (TCA) protein precipitation and acid hydrolysis as described by Esterhuizen and Downing (2008) with minor modifications. The hydrolysate was filtered using Ultrafree-MC® 0.22 µm centrifugal filtration units and the filtrate dried down using a Savant SpeedVac® Plus after which the dried residue was resuspended in 200 µL 20 mM HCl.

Samples of these amino acid extracts and hydrolysates were derivatised using propyl chloroformate and separated by UPLC (Waters Acquity Ultra Performance LC) on a Phenomenex EZ:faast AAA-MS column (250 x 2.0 mm) by gradient elution (0.00 min 68% B, 13.00 min 83% B, 13.01 min 68%B, 17.00 min 68% B) with a mobile phase composition of 10 mM ammonium formate in water (A) and 10 mM ammonium formate in methanol (B) (flow rate: 0.25mL.min^{-1} and column temperature: 35 °C). Derivatised samples of the hydrosylates were analysed using a Thermo Finnigan TSQ Ultra AM Quantum triple quadrupole mass spectrometer operating in positive ion mode with the following settings: ESI voltage set to 5000, nebulizing gas (N) at a flow rate of 40, vaporization temperature of 199 °C, capillary temperature at 270 °C and capillary and tube lens

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offsets set to 35 and 70, respectively. Collision-induced dissociation was achieved within the second quadrupole with parent ion-specific collision energies ranging from 10 to 13 and argon gas supplied at 1 mTorr.

Amino acids analysed were chosen based on their relevance to this study in terms of chemical structure, their position in *de novo* amino acid synthesis and their role in nitrogen assimilation. Detection and analysis of labelled amino acids were based on HPLC retention times and mass spectrometric detection (Thermo Finnigan TSQ Ultra AM Quantum operating in Q1 mode, no collision-induced dissociation of parent ion) of parent ion *m/z* $^{+1\ amu}$ in amino acids with a single nitrogen atom and both parent ions *m/z*$^{+1\ amu}$ and *m/z* $^{+2\ amu}$ in amino acids with two nitrogen atoms (*m/z* of parent ions are given in Table 3.1).

Table 3.1: A list of amino acids used in this study showing retention time (t_R) and parent ion *m/z* used in the detection and analysis of ^{15}N-labelled amino acids.

Amino acid	t_R (min)	Amino acid MW	Parent ion *m/z*		
			m/z (Derivative MW+1)	*m/z*$^{+1\ amu}$	*m/z*$^{+2\ amu}$
Arg	2.7	174.2	303	304	305
Gln	3.3	175.2	304	305	306
Ser	3.7	105.1	234	235	
Asn	3.8	132.1	243	244	245
β-Ala	5	89.1	218	219	
Ala	5.1	89.1	218	219	
Sar	5.8	89.1	218	219	
2,4-DAB	6.7	118.1	333	334	335
Orn	6.9	132.1	347	348	
BMAA	7.4	118.1	333	334	335
Lys	7.8	146.1	361	362	363
Asp	7.8	133.1	301	302	
Val	8.2	117.1	246	247	
Glu	8.3	147.1	318	319	

Analysis of BMAA and 2,4-diaminobutyric acid (2,4-DAB) were performed based on UPLC retention times and either parent ion 333 *m/z* or MS/MS detection (settings described above) of product ions *m/z* 245 and *m/z* 273 produced during collision-induced dissociation of parent ion *m/z* 333. Analysis of ^{15}N-labelled BMAA and 2,4-DAB were based on product ions *m/z* $^{+1\ amu}$ and *m/z* $^{+2\ amu}$ (*m/z* 246, 274 and *m/z* 247, 275).

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Analysis of the incorporation of ^{15}N into proteinogenic and non-proteinogenic amino acids was based on the relative abundance of ^{15}N-containing amino acids extracted from ^{15}N-exposed cultures, normalised against control cultures. Due to the nature of this study, the aim being to confirm the synthesis of BMAA in cyanobacteria based on the presence of labelled BMAA, no quantification of labelled or unlabelled amino acids was performed. Data represent the relative abundance of labelled amino acids based on LC/MS/MS peak areas, unless otherwise stated.

3.3 RESULTS AND DISCUSSION

3.3.1 Statistical analysis

We were interested to determine if exposure of cyanobacterial cultures to ^{15}N increased the ratio of $^{15}N/^{14}N$. Since the experiments were conducted with three replicates, we did not wish to make assumptions concerning normal distribution of the data and equal sample variances, and therefore decided to use a nonparametric sign test (Sprent and Smeeton, 2007) to test the statistical hypotheses. We counted the number of instances for different amino acids in which the $^{15}N/^{14}N$ ratio increased after a given time of exposure out of the total number of observations. The probability, p, of this occurrence is given by

$$p = \left(\frac{number\ of\ observations}{number\ of\ increases} \right) \left(1/2 \right)^{number\ of\ observations}$$

Where

$$\left(\frac{number\ of\ observations}{number\ of\ increases} \right) = \frac{(number\ of\ observations)!}{(number\ of\ increase)!(number\ of\ observations - number\ of\ increases)!}$$

Using this expression, p can then be compared to the area under the corresponding binomial distribution (which approximates a normal distribution) for $p<0.05$, $p<0.01$, $p<0.001$, etc.

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Nitrogen isotope ratios after 10 minutes of exposure to ^{15}N

a. Free amino acids: For an increase in $^{15}N/^{14}N$ ratios in free amino acids after 10 min of exposure, two alternative hypotheses were tested.

H_0 = the ratio of $^{15}N/^{14}N$ in Arg, Gln, Ser, Ala, Asp, Val, and Glu did not change after 10 min in cyanobacterial cultures fed with ^{15}N as compared to control cultures.

H_1 = the ratio of $^{15}N/^{14}N$ in Arg, Gln, Ser, Ala, Asp, Val, and Glu increased after 10 min in cyanobacterial cultures fed with ^{15}N as compared to control cultures.

For these hypotheses the test statistic $p = 4.47 \times 10^{-7}$, and therefore the null hypothesis was rejected at $p<0.0001$.

b. Bound amino acids: For an increase in $^{15}N/^{14}N$ ratios in bound amino acids after 10 min of exposure, two alternative hypotheses were tested.

H_0 = the ratio of $^{15}N/^{14}N$ in Arg, Ser, Val, Glu, BMAA, and DAB did not change after 10 min in cyanobacterial cultures fed with ^{15}N as compared to control cultures.

H_1 = the ratio of $^{15}N/^{14}N$ in Arg, Ser, and Val, Glu, BMAA, and DAB increased after 10 min in cyanobacterial cultures fed with ^{15}N as compared to control cultures.

For these hypotheses the test statistic $p = 3.62 \times 10^{-5}$, and therefore the null hypothesis was rejected at $p<0.001$.

Nitrogen isotope ratios after 24 hours of exposure to ^{15}N

a. Free amino acids: For an increase in $^{15}N/^{14}N$ ratios in free amino acids after 24 h of exposure, two alternative hypotheses were tested.

H_0 = the ratio of $^{15}N/^{14}N$ in Gln, Ser, Ala, Asp, Val, Glu and BMAA did not change after 24 h in cyanobacterial cultures fed with ^{15}N as compared to control cultures.

H_1 = the ratio of $^{15}N/^{14}N$ in Gln, Ser, Ala, Asp, Val, Glu and BMAA increased after 24 h in cyanobacterial cultures fed with ^{15}N as compared to control cultures.

For these hypotheses the test statistic $p = 3.62 \times 10^{-5}$, and therefore the null hypothesis was rejected at $p<0.001$.

b. Bound amino acids: For an increase in $^{15}N/^{14}N$ ratios in bound amino acids after 24 h of exposure, two alternative hypotheses were tested.

H_0 = the ratio of $^{15}N/^{14}N$ in Ala, Asp, Val, and Glu did not change after 24 h in cyanobacterial cultures fed with ^{15}N as compared to control cultures.

H_1 = the ratio of $^{15}N/^{14}N$ in Ala, Asp, Val, and Glu increased after 24 h in cyanobacterial cultures fed with ^{15}N as compared to control cultures.

For these hypotheses the test statistic $p = 6.87 \times 10^{-5}$, and therefore the null hypothesis was rejected at $p<0.001$.

Nitrogen isotope ratios after 48 hours of exposure to ^{15}N

a. Free amino acids: For an increase in $^{15}N/^{14}N$ ratios in free amino acids after 48 h of exposure, two alternative hypotheses were tested.

H_0 = the ratio of $^{15}N/^{14}N$ in Gln, Ser, Ala, Asp, and Glu did not change after 48 h in cyanobacterial cultures fed with ^{15}N as compared to control cultures.

H_1 = the ratio of $^{15}N/^{14}N$ in Gln, Ser, Ala, Asp, and Glu increased after 24 h in cyanobacterial cultures fed with ^{15}N as compared to control cultures.

For these hypotheses the test statistic $p = 3.05 \times 10^{-5}$, and therefore the null hypothesis was rejected at $p<0.001$.

b. Bound amino acids: For an increase in $^{15}N/^{14}N$ ratios in bound amino acids after 48 h of exposure, two alternative hypotheses were tested.

H_0 = the ratio of $^{15}N/^{14}N$ in Arg, Ser, Ala, Asp, Val, Glu, BMAA, and DAB did not change after 48 h in cyanobacterial cultures fed with ^{15}N as compared to control cultures.

H_1 = the ratio of $^{15}N/^{14}N$ in Arg, Ser, Ala, Asp, Val, Glu, BMAA, and DAB increased after 48 h in cyanobacterial cultures fed with ^{15}N as compared to control cultures.

For these hypotheses the test statistic $p = 1.43 \times 10^{-6}$, and therefore the null hypothesis was rejected at $p<0.0001$.

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3.3.2 ^{15}N labelling of free and protein-associated cellular amino acids

Both strains of cyanobacteria investigated, grown in the presence of $^{15}NH_4^+$ as sole nitrogen source, incorporated ^{15}N into free amino acids and proteins within 24 h of exposure. Exposure of nitrogen-deprived *Microcystis* to $^{15}NH_4^+$ resulted in an increase in the ^{15}N:^{14}N ratio of free amino acids Arg, Gln, Ser, Ala, Asp, Val, and Glu, relative to control samples, 10 min after exposure (Table 3.2). An increase in ^{15}N in amino acids such as arginine that, in cyanobacterial amino acid biosynthetic pathways, receives an amino group from a non-amino acid biomolecule, carbamoyl-phosphate, which in turn receives its amino group from glutamine or glutamate, suggests that ^{15}N was circulated throughout most newly synthesised molecules within 24 hours. However, no increase in ^{15}N-labelled BMAA or 2,4-DAB was seen within the same time frame of ^{15}N increase in free proteinogenic amino acids (Table 3.2). Interestingly, exposure of the 24 h nitrogen-deprived *Microcystis* cultures to NH_4^+ resulted in a decrease in free cellular BMAA within 10 minutes of exposure, compared with exposure of the same culture to NO_3^- resulting in a delayed decrease in free cellular BMAA (Figure 3.1).

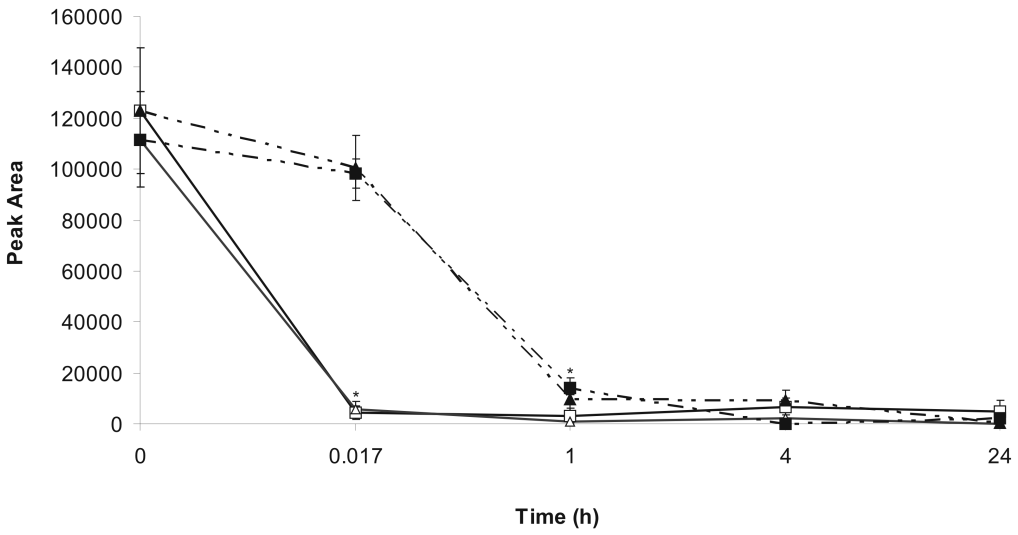

Figure 3.1: The disappearance of free cellular BMAA upon exposure of nitrogen starved *Microcystis* PCC7806 to $^{15}NH_4^+$ (solid line) and $^{14}NO_3^-$ (broken line), respectively. The graph depicts the peak areas of detection using LC/MS/MS analysis of product ions 245 *m/z* ($^{14}NO_3^-$, solid triangles and $^{15}NH_4^+$, open squares) and 273 *m/z* ($^{14}NO_3^-$, solid squares and $^{15}NH_4^+$, open triangles) produced upon collision-induced dissociation (CID) of the BMAA parent ion 333 *m/z*. Error bars denote the standard deviation of the mean (n=3), where (*) indicates a significant ($p< 0.05$) decrease compared to control samples.

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The delay in the decrease of free cellular BMAA on exposure to nitrate, if attributable to the requirements for nitrate reduction by the cyanobacteria, and the relatively rapid removal of free BMAA on exposure to ammonium, suggest a direct effect of ammonium on BMAA. The absence of ^{15}N labelled BMAA and 2,4-DAB following 24 h exposure of *Microcystis* to ^{15}N suggests that these amino acids are not synthesised by transamination reactions during primary synthesis of proteinogenic amino acids. This is supported by the fact that no increase in ^{15}N content of the amino acid sarcosine, which is not synthesised during primary amino acid synthesis, was observed within the same time frame (Figure 3.2). When comparing data from this study with the biosynthetic pathway proposed by Brenner *et al.* (2003), the proposed pathway seems unlikely because it relies on the presence of ammonia whereas our data show the production of BMAA in the absence of available nitrogen, and the disappearance of BMAA with the addition of ammonia.

Table 3.2: The relative increase in the ratio ($Ion^{+1\ amu}$: Ion) in ^{15}N fed cultures (n=3) normalised against ^{14}N fed control cultures of selected free (a) and protein associated (b) cellular amino acids during 24 hours exposure of nitrogen starved *Microcystis* PCC7806 cultures to $^{15}NH_4^+$. Note, incorporation of heavy nitrogen into free cellular BMAA seen only during longer term exposure (see Figure 3.3).

(a)

Amino Acids	Exposure time (h)												Nitrogen starvation			
	0.017			1			4			24				48		
Arg	0.008	±	0.458	0.035	±	0.458	-		-	-		-		0.189	±	0.004
Gln	5.402	±	0.436	20.686	±	0.436	8.953	±	0.784	4.752	±	4.238		0.153	±	0.035
Ser	0.293	±	0.037	1.156	±	0.037	1.415	±	0.265	-		-		0.202	±	0.042
Asn	0.139	±	0.242	0.550	±	0.242	0.308	±	0.533	-		-		0.890	±	0.273
Ala	1.113	±	0.026	1.090	±	0.026	1.445	±	0.174	1.495	±	0.525		0.174	±	0.038
Asp	2.089	±	0.055	8.660	±	0.055	8.376	±	2.273	5.179	±	1.613		0.106	±	0.010
Val	0.482	±	0.056	0.994	±	0.056	1.758	±	0.236	1.395	±	0.409		0.309	±	0.117
Glu	2.139	±	0.017	7.272	±	0.017	11.055	±	0.300	7.829	±	1.286		0.156	±	0.021
2,4-DAB	-		-	-		-	-		-	-		-		-		-
BMAA	-		-	-		-	-		-	-		-		-		-

(b)

Amino Acids	Exposure time (h)												Nitrogen starvation			
	0.017			1			4			24				48		
Arg	0.067	±	0.057	0.005	±	0.031	-		-	-		-		0.133	±	0.050
Gln	-		-	-		-	-		-	-		-		-		-
Ser	0.008	±	0.014	0.002	±	0.010	0.018	±	0.020	0.211	±	0.052		0.244	±	0.042
Asn	-		-	-		-	-		-	-		-		-		-
Ala	-		-	0.004	±	0.004	0.033	±	0.001	0.205	±	0.047		0.246	±	0.027
Asp	0.011	±	0	0.020	±	0.020	0.152	±	0.010	0.263	±	0.027		0.251	±	0.028
Val	0.013	±	0.003	0.023	±	0.009	0.047	±	0.002	0.217	±	0.052		0.261	±	0.026
Glu	0.032	±	0.001	0.036	±	0.032	0.063	±	0.004	0.280	±	0.048		0.315	±	0.027
2,4-DAB	0.062	±	0.020	-		-	0.037	±	0.013	-		-		0.005	±	0.004
BMAA	0.062	±	0.021	-		-	0.033	±	0.023	-		-		0.014	±	0.012

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As noted above, a significant (p<0.0001) increase in the ^{15}N:^{14}N ratio , Asp, Val, and Glu extracted from *Microcystis* proteins was seen after 24 h exposure to $^{15}NH_4^+$ (Table 3.2b), and this ratio remained elevated relative to control samples, following 24 h nitrogen starvation of these cultures. It is difficult to explain the observed coincident fluctuation in ^{15}N:^{14}N of protein-associated 2,4-DAB and BMAA over time in 24 h starved cultures supplemented with nitrogen (Table 3.2), although covalent modification of protein-associated 2,4-DAB or BMAA may explain this observation. It is striking, however, that the ^{15}N:^{14}N ratio in protein-associated BMAA increased within 10 minutes of exposure to ^{15}N.

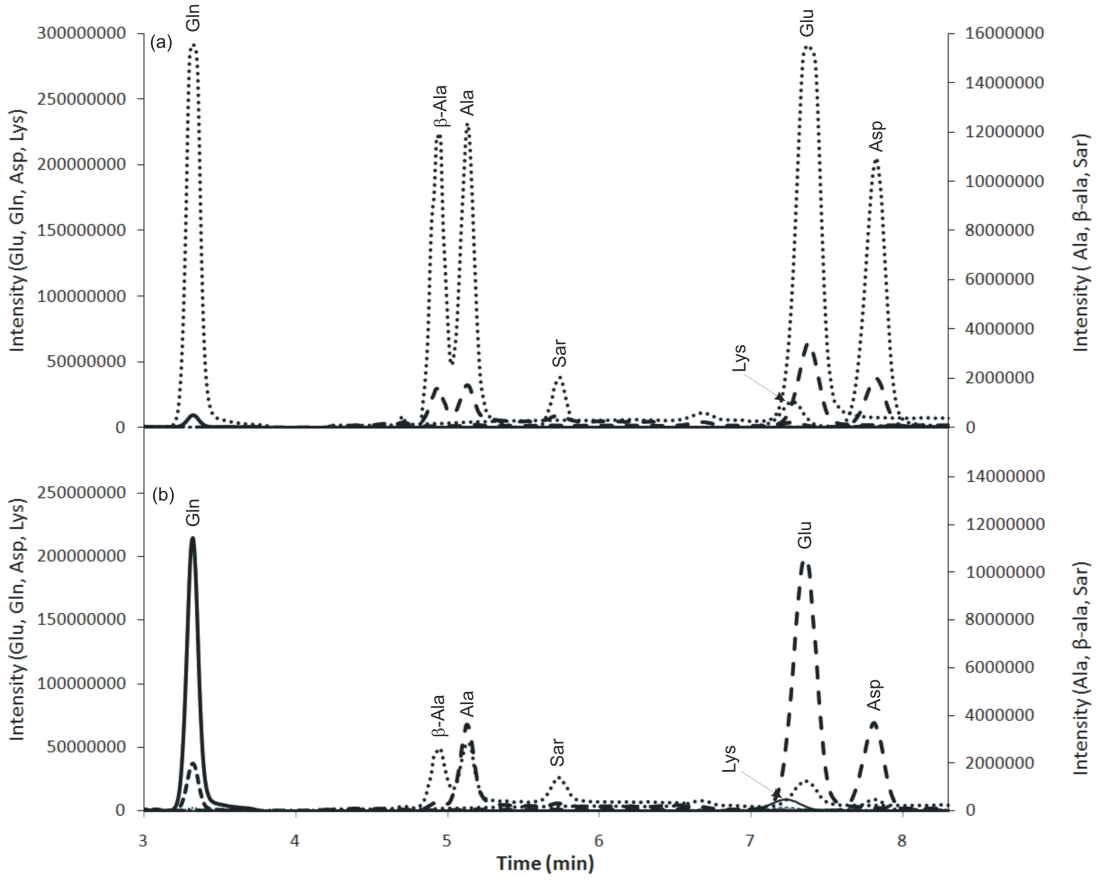

Figure 3.2: Example LC/MS chromatograms depicting the qualitative analysis of the signal intensities obtained during detection of $m/z^{+0\ amu}$ (dotted line) $m/z+^{1\ amu}$ (dashed line) and $m/z^{+2\ amu}$ (solid line) parent ions, show the increase in ^{15}N content of selected amino acids in *Microcystis* PCC7806 cultures grown with $^{15}NH_4^+$ as sole nitrogen source (b) compared to the presence of ^{15}N in amino acids extracted from *Microcystis* PCC7806 cultures grown with $^{14}NO_3^-$ as sole nitrogen source (a).

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3.3.3 Release of free intracellular BMAA upon nitrogen starvation

No free or protein associated BMAA was detected in *Synechocystis* after growth for 10 days with either excess NO_3^- or excess NH_4^+ as the sole nitrogen source. Subsequent nitrogen deprivation resulted in the appearance of either free cellular ^{14}N BMAA or free cellular ^{15}N BMAA after 48 h, depending on the isotope of nitrogen fed prior to deprivation. Free cellular BMAA content continued to increase over 120 h during nitrogen deprivation (Figure 3.3). An increase in ^{15}N-labelled BMAA was detected in nitrogen-deprived *Synechocystis* cultures previously grown on $^{15}NH_4^+$, with the ratio of ^{15}N BMAA to ^{14}N BMAA observed in control cultures representing the natural abundance of the isotope (Figure 3.4).

As with *Synechocystis*, free cellular ^{14}N and ^{15}N BMAA was detected upon nitrogen deprivation of *Microcystis* cultures supplemented with either $^{14}NO_3^-$ or $^{15}NH_4^+$ as sole nitrogen sources over 90 days prior to starvation. However, BMAA was only detected following 8 days of deprivation and not 48 hours as observed in the *Synechocystis* culture after growth on nitrogen for 10 days.

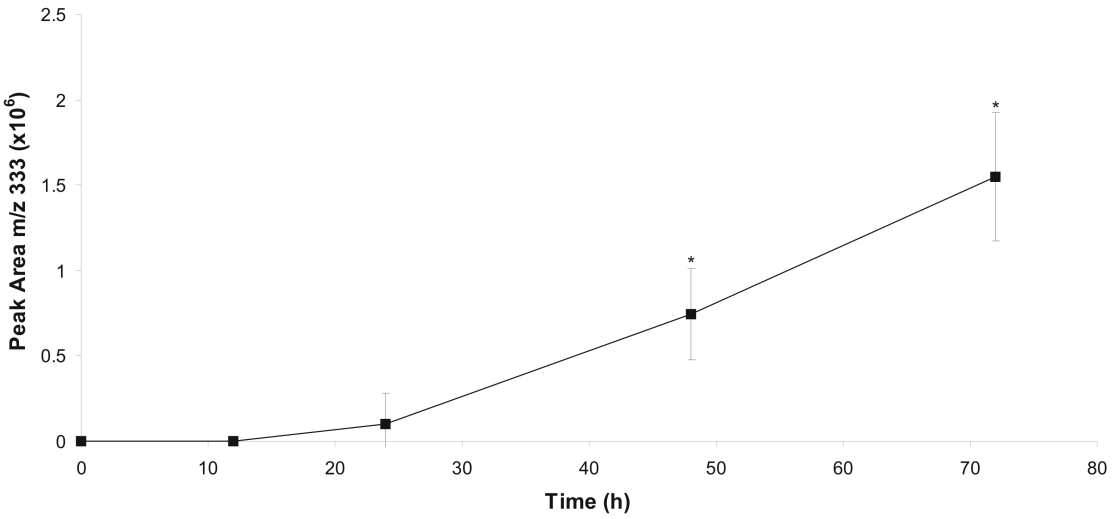

Figure 3.3: The increase in free cellular BMAA in *Synechocystis* J341 following 48 hours nitrogen starvation of cultures grown with NO_3^- as sole nitrogen source. The graph depicts peak areas of LC/MS detection of BMAA parent ion 333 *m/z*, where (*) indicates a significant ($p < 0.05$) increase. Error bars denote the standard deviation of the mean (n=3).

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These data suggest that, over and above strain or species specific variation, the rate of BMAA production upon nitrogen deprivation may be determined by the duration of growth in the presence of excess nitrogen or the nutritional status prior to starvation. These data confirm the production of BMAA by cyanobacteria from raw starting material ($^{15}NH_4^+$) and show that BMAA is produced within cyanobacteria in response to nitrogen starvation. The data from this study may serve to explain why many investigators have not detected BMAA in cyanobacterial samples, as laboratory cultures are generally not maintained under nitrogen limitation.

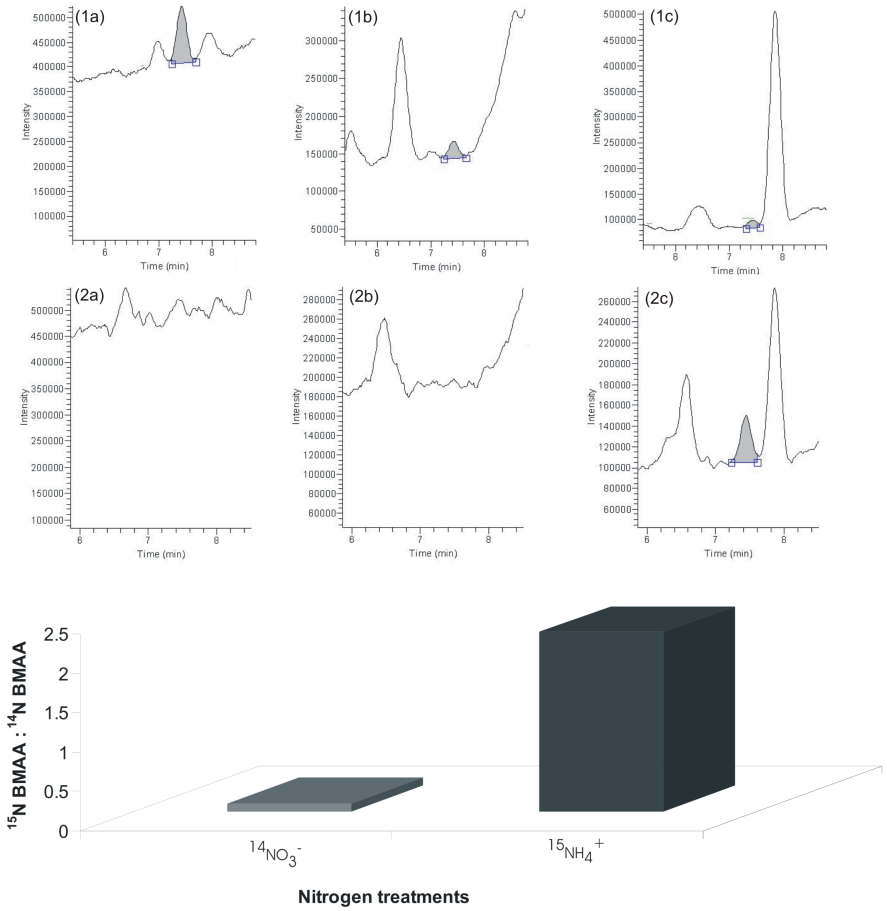

Figure 3.4: Upper pane: The chromatograms show the LC/MS detection of native BMAA parent ion 333 *m/z* (a) and ^{15}N-containing BMAA parent ions 334 *m/z* (b), and 335 *m/z* (c). These chromatograms represent free cellular BMAA extracted from *Synechocystis* J341 grown with $^{14}NO_3^-$ (1) or $^{15}NH_4^+$ (2) as sole nitrogen sources, following 72 hours of nitrogen starvation. Chromatograms 2a-c clearly show the replacement of light BMAA by ^{15}N BMAA in cultures grown with $^{15}NH_4^+$, with control cultures (1a-c) showing a natural abundance of ^{15}N BMAA which corresponds to the natural abundance of this isotope. Lower pane: The ratio of heavy to light BMAA during nitrogen starvation after growth on either $^{14}NO_3^-$ or $^{15}NH_4^+$ as sole nitrogen source (n=3).

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3.4 CONCLUSION

This study constitutes the first report of *de novo* synthesis of stable isotope labelled BMAA by an axenic unicellular non-nitrogen fixing cyanobacterial culture, *Microcystis* PCC7806. BMAA is not synthesised along with proteinogenic amino acids during primary transamination reactions involving glutamate or glutamine, nor during transamination reactions by other proteinogenic amino acids. Furthermore, BMAA is not produced within the time frame of *de novo* synthesised proteinogenic amino acids. Free cellular BMAA is produced upon nitrogen deprivation suggesting that BMAA is produced as a breakdown product or released from a bound form. However, the absence of a negative correlation between protein-associated and free BMAA indicates that it is produced specifically upon nitrogen deprivation as opposed to being released from a reservoir. This in turn suggests that it may have a function in nitrogen metabolism for unicellular non-nitrogen fixing cyanobacteria.

These experiments confirm the synthesis of BMAA by cyanobacteria. This study also highlights the complexity of the possible function of BMAA in these organisms in addition to providing the first information regarding environmental modulation of intracellular BMAA pools within cyanobacteria, an area that requires further investigation.

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The cyanobacterial origin of BMAA was confirmed via the detection of isotopically labelled BMAA produced from labelled ammonia by an axenic culture. BMAA is specifically produced by cyanobacteria under nitrogen deprivation and under nitrogen-limited conditions free intracellular BMAA disappears rapidly upon the addition of combined nitrogen to the culture medium, with no concomitant increase in protein associated BMAA. The inverse relationship between nitrogen availability and BMAA was also observed in natural environmental samples (See Appendix B). This strongly suggests that BMAA metabolism in cyanobacteria is closely associated with the cellular nitrogen status.

In order to understand the role of BMAA in cyanobacteria, the effects of this amino acid on growth and nitrogen controlled processes were investigated.

CHAPTER 4: THE EFFECT OF EXOGENOUS β-*N*-METHYLAMINO-L-ALANINE ON THE GROWTH OF *SYNECHOCYSTIS* PCC6803

ABSTRACT

β-*N*-Methylamino-L-alanine (BMAA), a nonproteinogenic amino acid, has been detected in a range of cyanobacteria, including terrestrial, aquatic, free living and endosymbiotic species. The widespread occurrence of cyanobacteria in the environment raises concerns regarding the ecological and toxicological impact of BMAA, and consequently, studies have focussed extensively on the toxicity and environmental impact of BMAA, while no research has addressed the ecophysiological or metabolic role of the compound in cyanobacteria. In this study, both the uptake of exogenous BMAA by and the effect of exogenous BMAA on the growth of *Synechocystis* PCC6803 were investigated. BMAA was rapidly taken up by the non-diazotrophic cyanobacterium *Synechocystis* PCC6803 in a concentration dependent manner. The presence of exogenous BMAA resulted in a substantial and concentration-dependent decrease in cell growth and the substantial loss of photosynthetic pigmentation. Similar effects were seen in the presence of the non-proteinogenic amino acid, 2,4-diaminobutyric acid but to a lesser degree than that of BMAA. The effects were reversed when light was decreased from 16 to 10 µmol m^{-2} s^{-1}. Control cultures grown in the presence of L-arginine, L-asparagine, L-glutamate and glycine showed normal or slightly increased growth with no change in pigmentation. The decrease in growth rate coupled to bleaching indicates that BMAA may induce chlorosis in the presence of adequate photosynthetic radiation suggesting a connection between BMAA and the induction of conditions, such as nitrogen or sulphur depletion, that result in growth arrest and the induction of chlorosis.

4.1 INTRODUCTION

β-*N*-Methylamino-L-alanine (BMAA) is a non-proteinogenic amino acid produced by a range of ecologically and taxonomically diverse cyanobacteria, including freshwater, terrestrial and marine species, and is present within cells in both free and

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protein-associated forms (Banack *et al.,* 2007; Cox *et al.,* 2005; Esterhuizen and Downing, 2008). No correlation exists between BMAA production and taxonomic groups or geographical regions (Cox *et al.,* 2005; Esterhuizen and Downing, 2008). The compound, a slow acting neurotoxin linked to the neurodegenerative disease Amyotrophic Lateral Sclerosis–Parkinsonism–Dementia Complex (Kurland, 1972; Spencer *et al.,* 1987) first isolated from *Cycas* spp., was later linked to cyanobacteria when it was isolated from the cycad root endosymbiotic cyanobacterium, *Nostoc* (Cox *et al.,* 2003).

The link between BMAA and environmentally ubiquitous cyanobacteria and neurodegeneration together with the knowledge that BMAA bioaccumulates and significantly biomagnifies within ecosystems (Cox *et al.,* 2003) has resulted in research being focused on the toxicology and environmental impact of the compound rather than its biosynthesis or function in the producing cyanobacteria. The question surrounding the role of BMAA in cyanobacteria remains unanswered.

Uptake of exogenous BMAA by *Arabidopsis* results in retarded root growth and cotyledon opening whilst stimulating hypocotyl elongation of light-grown seedlings (Brenner *et al.,* 2000). Furthermore, uptake of exogenous BMAA causes a significant decrease in the specific activity of oxidative stress enzymes; superoxide dismutase, peroxidase, glutathione reductase and catalase and a non-significant decrease in specific activity of glutathione *S*-transferase in *Ceratophyllum demersum* (Esterhuizen *et al.,* 2011a,c). Neither the uptake nor the effects of exogenous BMAA in cyanobacteria have been investigated, an understanding of which may aid in the elucidation of the possible roles of the compound in these organisms.

Substantial variance in BMAA content and the ratio of free to protein-associated BMAA of extracts have been observed in several laboratory cultured cyanobacterial strains (Cox *et al.,* 2005; Esterhuizen and Downing, 2008). This suggests that BMAA production may be a function of growth phase and/or environmental and nutritional conditions or stresses and that such fluctuations in the intracellular BMAA pool may serve as a

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regulatory mechanism for certain metabolic processes. Furthermore, a number of cyanobacteria, including *Anabaena* sp. and *Synechocystis* PCC6803, are capable of photoheterotrophic growth (Anderson and McIntosh, 1991; Montesinos *et al.,* 1995) with amino acids, L-arginine (Arg), L-asparagine and L-glutamine, or urea as a nitrogen source (Flores and Herrero, 1994) and Arg as a carbon source in *Synechocystis* sp. strain PCC6308 (Weathers *et al.,* 1978). Amino acid transport systems in cyanobacteria have only been characterised in detail for strains *Synechocystis* sp. strain PCC6803 and *Anabaena* sp. strain PCC7120. *Synechocystis* PCC6803 contains three amino acid transport systems for the uptake of basic amino acids (including glutamine), neutral amino acids (excluding glutamine) and glutamate and glutamine. Similarly, one basic transport system and two systems with overlapping specificities for neutral amino acids have been identified in *Anabaena* PCC7120 (Montesinos *et al.,* 1995). The study of amino acid uptake in cyanobacteria has been most commonly achieved through the use of ^{14}C-amino acids, with the cellular incorporation of amino acids based on the detection of the radioactive label in whole cells collected through filtration following amino acid exposure (Montesinos *et al.,* 1995; Ping and McAuley, 1990). In order to elucidate the biological function(s) of BMAA in cyanobacteria, we investigated the uptake of exogenous BMAA by the uni-cellular cyanobacterium *Synechocystis* PCC6803 using LC/MS analysis of cellular extracts prepared following the exposure of cultures to exogenous BMAA. In addition, we investigated the effect of exogenous BMAA on the growth of this strain.

4.2 METHODS

4.2.1 Strains and culture conditions

A uni-algal *Synechocystis* PCC6803 (non-diazotrophic) culture was obtained from the IGB (Leibniz Institute of Freshwater Ecology and Inland Fisheries), Berlin, Germany. This strain was selected because it has natural free BMAA levels below the limit of detection and protein-associated levels below the limit of quantification (Esterhuizen-Londt *et al.,* 2011b).

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Cultures were maintained in BG-11 (Rippka, 1988) under sterile conditions at a temperature of 23 °C (±1°C) with constant illumination at a light intensity of 16 µmol m^{-2} s^{-1} (Triton Dayglo©, Cleveland, OH, USA). Culture purity was regularly monitored microscopically.

4.2.2 Uptake of exogenous BMAA

Mid-log cultures were exposed to exogenous BMAA (Sigma, St. Louis, MO, USA) over 96 h at concentrations of 0.05, 0.5 and 5.0 µM. Cells from a mid-log *Synechocystis* PCC6803 culture in BG-11 were harvested by centrifugation (5,000 x *g* for 10 min) and re-suspended in fresh BG-11 media, after which the culture was aliquoted into replicate Erlenmeyer flasks, which were then supplemented with exogenous BMAA as described. Samples were taken at 10 min after exposure, followed by 24-h intervals.

4.2.3 Analysis of cellular BMAA content

Samples were centrifuged at 8,000 x *g* for 10 min using a Beckman Avanti J-20 centrifuge after which cell pellets were lyophilised overnight using a VirTis freeze dryer (−42.6°C, 170 mTorr vacuum). Free and protein-associated BMAA were subsequently extracted from the dried cell pellets (7–10 mg) using trichloroacetic acid protein precipitation and acid hydrolysis as described by Esterhuizen and Downing (2008). Free and protein-associated BMAA fractions were derivatised and quantified using EZ:faast™-Free (Physiological) Amino Acid Analysis by high-performance liquid chromatography–mass spectrometry (Phenomenex, Torrance, CA, USA), an amino acid analysis procedure based on solid-phase extraction of free amino acids followed by derivatisation of BMAA by modification of both amino and carboxyl groups with a chloroformate derivative. Free and protein-associated cellular BMAA were analysed using a Shimadzu LC/MS-2010 EV.

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Derivatised samples (injection volume of 1 µL) were separated using a Phenomenex EZ:faast™ AAA-MS 250 x 2.00 mm amino acid analysis column (Phenomenex, Torrance, CA, USA) by gradient elution (0.00 min 68% B, 13.00 min 83% B, 13.01 min 68% B, 17.00 min 68% B) with a mobile phase composition of 10 mM ammonium formate (Separations, South Africa) in water (Merck, Darmstadt, Germany) (A) and 10 mM ammonium formate in methanol (Merck, Darmstadt, Germany) (B) (flow rate 0.25 mL min^{-1} and column temperature 35 °C). BMAA was quantified using electron spray ionisation (ESI) positive ion mode mass spectrometry with an ESI ion source temperature of 250 °C, scan range of 100–650 *m/z* and detector voltage of −1.45 kV. Nitrogen was used as nebulising gas at a flow rate of 1.50 L min^{-1} with a block temperature of 200 °C.

4.2.4 Analysis of growth rate

Mid-log cultures were exposed to exogenous BMAA and a selection of control amino acids at concentrations of 4.2 and 42 µM over 192 h. Cells from a mid-log *Synechocystis* PCC6803 culture in BG-11 were harvested by centrifugation (5,000 x *g* for 10 min) and re-suspended in fresh BG-11 media, after which the culture was aliquoted into replicate Erlenmeyer flasks, which were then supplemented with respective amino acids at concentrations described above. Control amino acids included L-asparagine (Sigma Aldrich, St. Louis, MO, USA), L-arginine (Sigma Aldrich, Tokyo, Japan), L-glutamate (Fluka, Lyon, France), glycine (Fluka, Buchs, Switzerland) and L-2,4-diaminobutyric acid (2,4-DAB) (Fluka, Bornem, Belgium). In addition, a negative control of unsupplemented BG-11 was included. Samples were taken at 10 min after exposure, followed by 24-h intervals. Growth rate was determined by means of a Beckman Coulter Cytomics FC 500 using an argon ion laser at an excitation wavelength of 488 nm. Dot plots of side scatter (log) vs. forward scatter (log) were used to gate on the population of *Synechocystis* PCC6803 and calibration beads for quantification.

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Synechocystis PCC6803 fluorescence was detected in channel FL4 (red fluorescence) with a minimum of 20,000 events, which were calibrated against Beckman Coulter Flow-Count™ Fluorospheres (Beckman Coulter, Fullerton, CA, USA) using fluoro-channel FL1 (green fluorescence) and used to determine photosynthetic viability and chlorosis.

4.2.5 Statistical analysis

Statistical significance of variance was determined by means of Student's *t* test (two-sample assuming unequal variances) using Statistica 9.0 (StatSoft Inc., Tulsa, OK, USA).

4.3 RESULTS

4.3.1 Uptake of exogenous BMAA by *Synechocystis* PCC6803

Synechocystis PCC6803 showed rapid and substantial uptake of exogenous BMAA in a concentration-dependent manner (Figure 4.1). Figures 4.1 and 4.2 indicate the change in free and protein-associated cellular BMAA fractions, respectively, normalised against control sample BMAA content. A significant (p<0.05) increase in free intracellular BMAA (Figure 4.1) was seen in cultures exposed to 0.5 and 5.0 µM exogenous BMAA within 10 min of exposure, with no significant increase in free cellular BMAA in cultures exposed to 0.05 µM exogenous BMAA. Within 24-h following exposure, a significant (p<0.05) decrease (relative to the 10 min values) in free BMAA fractions was seen in the presence of both 0.5 and 5.0 µM exogenous BMAA, after which there were no significant changes in free BMAA in cultures exposed to either concentration. Throughout the 96-h exposure, free cellular BMAA concentrations in cultures exposed to 5.0 µM BMAA were significantly (p<0.05) higher compared to cultures exposed to 0.5 µM exogenous BMAA.

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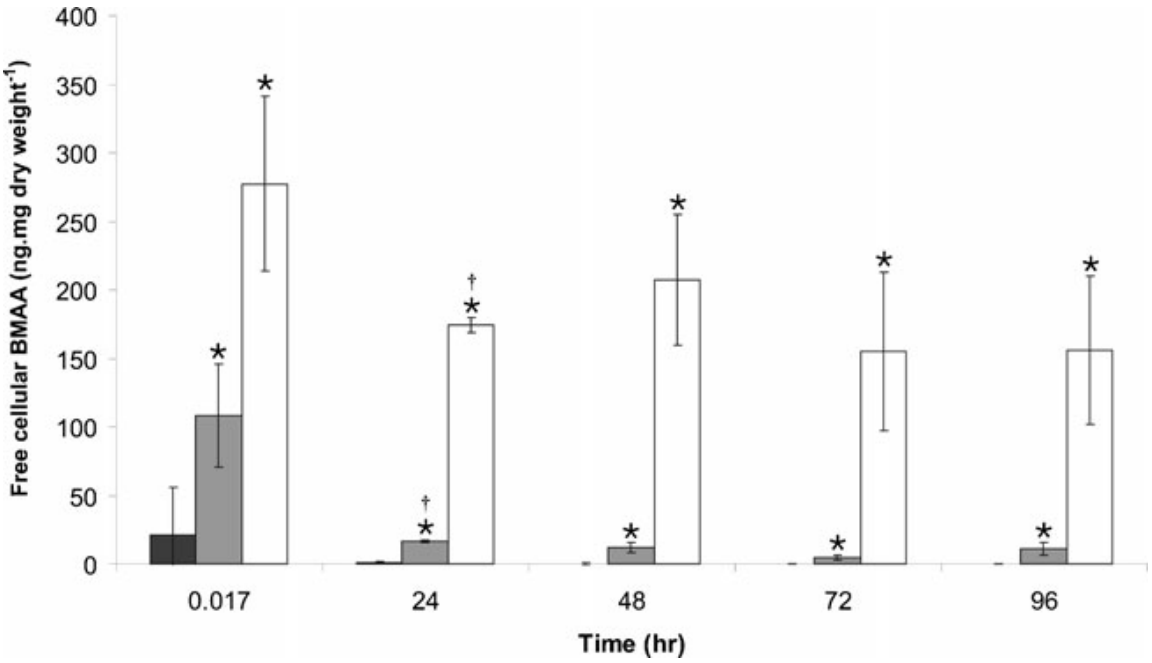

Figure 4.1: Total free cellular BMAA in *Synechocystis* PCC6803during 96 h exposure to three concentrations of exogenous BMAA, normalised against control samples; 0.05 µM BMAA (solid dark grey bars); 0.5 µM BMAA (solid light grey bars); 5.0 µM BMAA (open bars). Error bars denote the standard deviation of the mean (n=3). A significant (p<0.05) change in free cellular BMAA compared to a ten-fold lower exogenous BMAA concentration is indicated by (*), where a significant change in free cellular BMAA within 24 h of previous sample (of the same concentration) is indicated by a "dagger".

Exposure to 0.05 µM BMAA resulted in an insignificant increase in protein-associated BMAA within 10 min, followed by a subsequent decrease and complete disappearance within 48 h (Figure 4.2). Exposure to 0.5 µM exogenous BMAA resulted in a significant (p<0.05) initial increase in protein-associated BMAA within 10 min of exposure, followed by a decrease in protein-associated BMAA, with a significant decrease seen after 48 h and complete disappearance within 96 h (Figure 4.2). Similarly exposure to 5 µM BMAA resulted in a significant (p<0.05) initial increase in protein-associated BMAA within 10 min, followed by a significant (p<0.05) decrease within 24 h and complete disappearance within 72-h following exposure (Figure 4.2).

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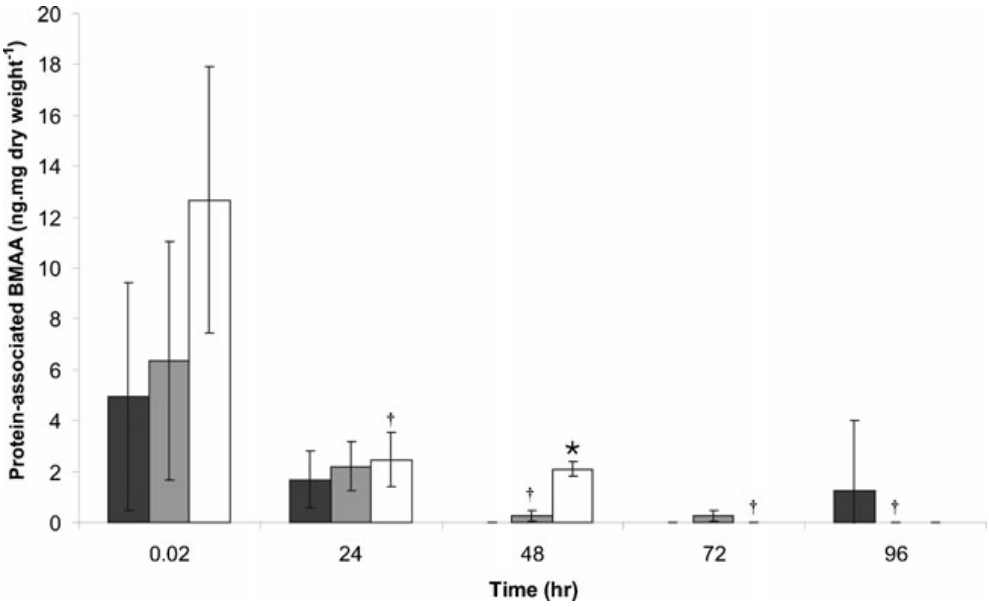

Figure 4.2: Total protein-associated cellular BMAA in *Synechocystis* PCC6803 after 96 h exposure to three concentrations of exogenous BMAA normalised against control samples; 0.05 µM BMAA (solid dark grey bars); 0.5 µM BMAA (solid light grey bars); 5.0 µM BMAA (open bars). Error bars denote the standard deviation of the mean (n=3) with a significant (p<0.05) change in protein-associated BMAA compared to a ten-fold lower exogenous BMAA concentration indicated by (*), where a significant change in free cellular BMAA within 24 h of previous sample (of the same concentration) is indicated by a "dagger".

4.3.2 The effect of exogenous BMAA on the growth of *Synechocystis* PCC6803

Figure 4.3 depicts example flow cytometry plots used in the analysis of cell growth. The presence of exogenous BMAA in the growth medium resulted in a concentration dependent decrease in the growth of *Synechocystis* PCC6803 (Figure 4.4). After 48 h exposure to 4.2 and 42 µM BMAA, significant decreases (p<0.05) in growth were observed. Significantly reduced growth (p<0.05) was still observed after 192 h in the presence of both 4.2 and 42 µM exogenous BMAA. The decrease in growth in the presence of exogenous BMAA can be attributed to the induction of chlorosis and subsequent cell lysis depicted in Figures 4.4 and 4.5.

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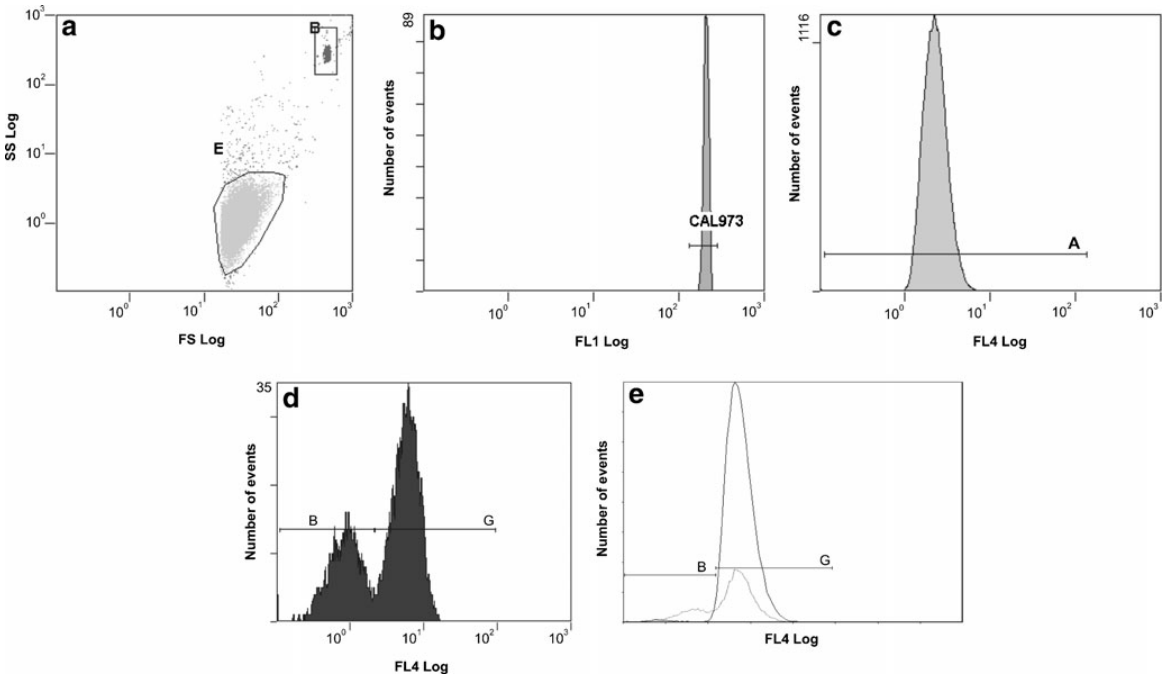

Figure 4.3: Example dot plot (a) of side scatter (log) vs. forward scatter (log) used to gate on the population of *Synechocystis* PCC6803 in region E and the calibration beads in region B and example histograms for green fluorescence (b) origination from the gated population of calibration beads and red fluorescence (c) from the gated population of *Synechocystis* PCC6803 (A). An example histogram (d) showing red fluorescence gated on *Synechocystis* PCC6803 cells in region E of the dot plot (a). Two populations B and G indicated in histogram (d) were seen with B corresponding to chlorotic *Synechocystis* PCC6803 cells with greatly reduced fluorescence and G corresponding to the actively growing *Synechocystis* PCC6803 cell population with a high fluorescence. Histogram (e) shows an overlay plot of a control culture (solid line) and a culture exposed to 5 µM exogenous BMAA (dotted line), clearly showing a decrease in photosynthetically active cells (G) and an increase in chlorotic cells (B).

In the presence of lower exogenous BMAA concentrations (4.2 µM), growth was retarded but not inhibited, with a positive yet reduced mean growth rate constant after 72 h, as shown in Figure 4.6. A ten-fold increase in exogenous BMAA concentration resulted in the inhibition of growth (Figure 4.4b) and an overall negative growth rate after 24 h (Figure 4.6). A comparable, substantial and significant decrease (p<0.05) in growth rate and mean growth rate constant was observed in the presence of 42 µM 2,4-DAB (Figure 4.4b and Figure 4.6), an isomer of BMAA (Banack *et al.,* 2010). The presence of both 4.2 and 42 µM exogenous BMAA also resulted in a noticeable loss of pigmentation within 48 h, with chlorosis also occurring in the presence of 42 µM 2,4-DAB, however to a lesser extent (data not shown).

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Following 192 h of exposure to exogenous amino acids, the light intensity was reduced from 16 to 10 µmol m^{-2} s^{-1}, which resulted in the recovery of growth and photosynthetic pigmentation in the presence of 4.2 and 42 µM exogenous BMAA, although growth in the presence of exogenous BMAA was still lower than control samples (Figure 4.7).

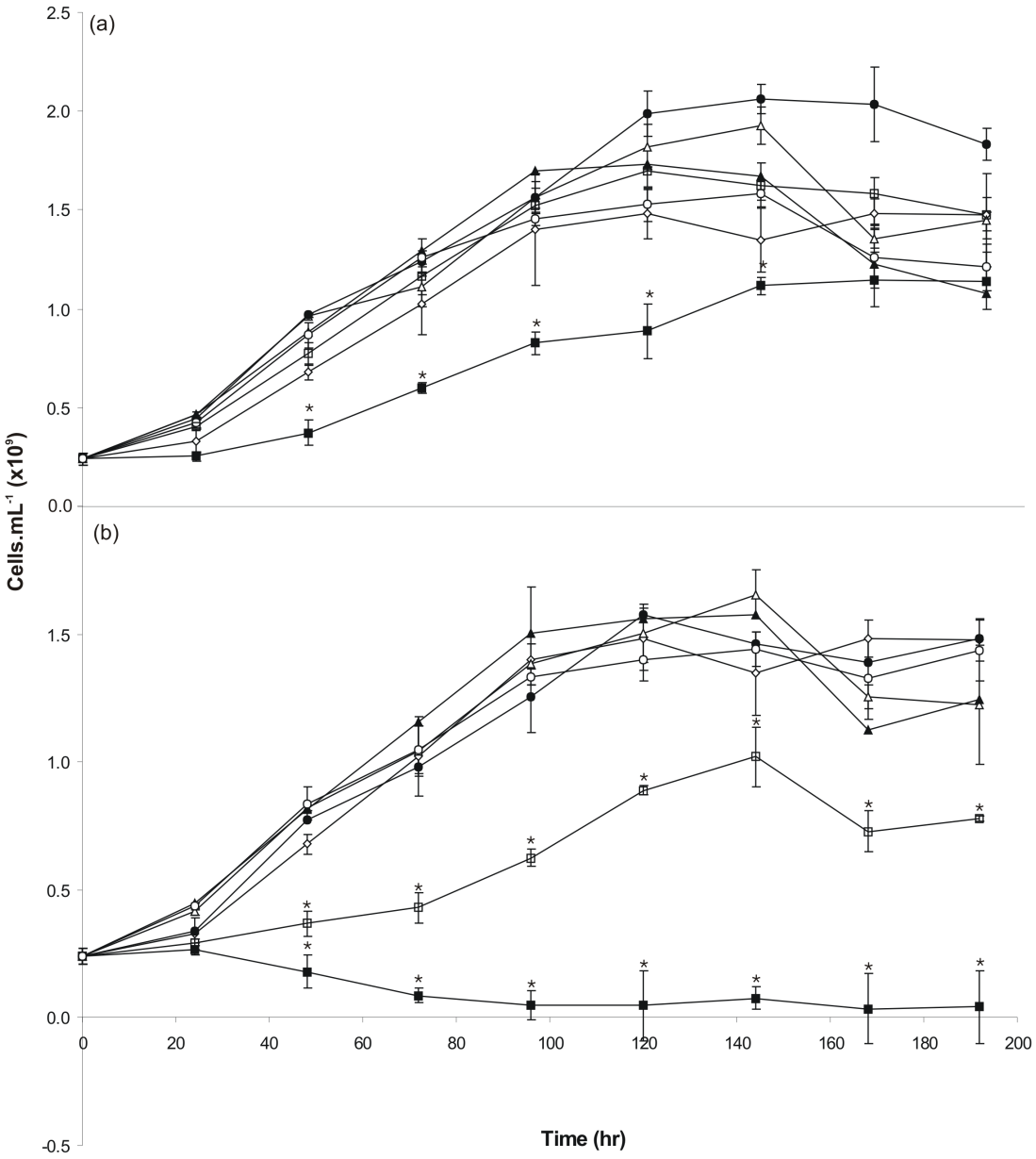

Figure 4.4: The effect of 4.2 µM (a) and 42 µM (b) exogenous amino acids on the growth of *Synechocystis* PCC6803 measured over 192 h. Negative control (open diamonds); BMAA (filled squares); Arg (filled circles); L-Asn (open triangles); Glu (filled triangles); Gly (open circles); 2,4-DAB (open squares). Error bars denote the standard deviation of the mean (n=5) with a significant (p<0.05) decrease in growth compared to control cultures indicated by (*).

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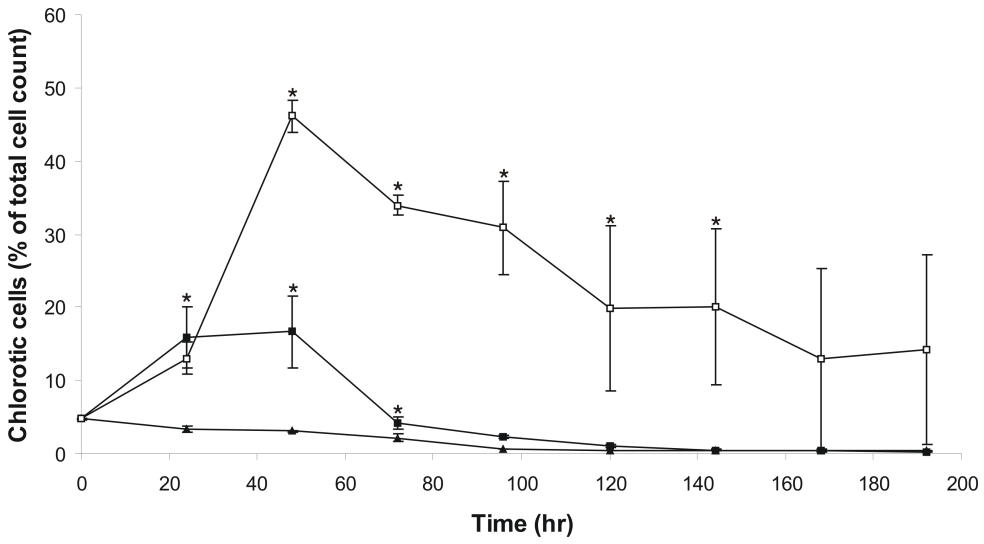

Figure 4.5: The increase in chlorotic cells and the subsequent decrease due to cells lysis in *Synechocystis* PCC6803 cultures exposure to 4.2 µM BMAA (filled squares) and 42 µM BMAA (open squares) compared to negative control cultures (filled triangles). Error bars denote the standard deviation of the mean (n=3) with a significant (p<0.05) increase in chlorosis compared to control cultures indicated by (*).

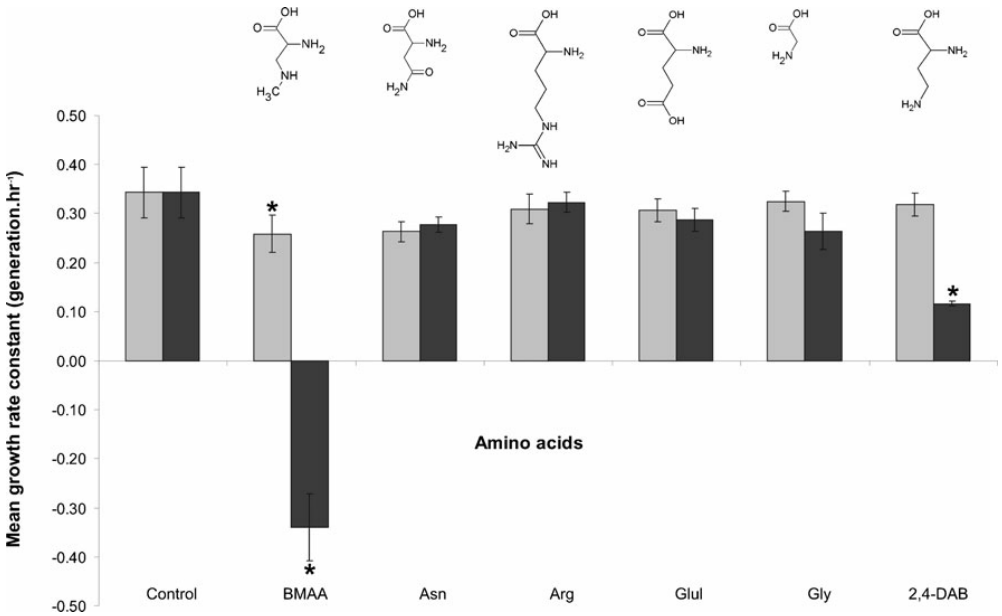

Figure 4.6: Mean growth rate constants of *Synechocystis* PCC6803 calculated after 72 h exposure to 4.2 µM (solid light grey bars) and 42 µM (solid dark grey bars) exogenous amino acids. Error bars denote the standard deviation of the mean (n=5) with a significant (p<0.05) decrease in the mean growth rate constant compared to control cultures indicated by (*).

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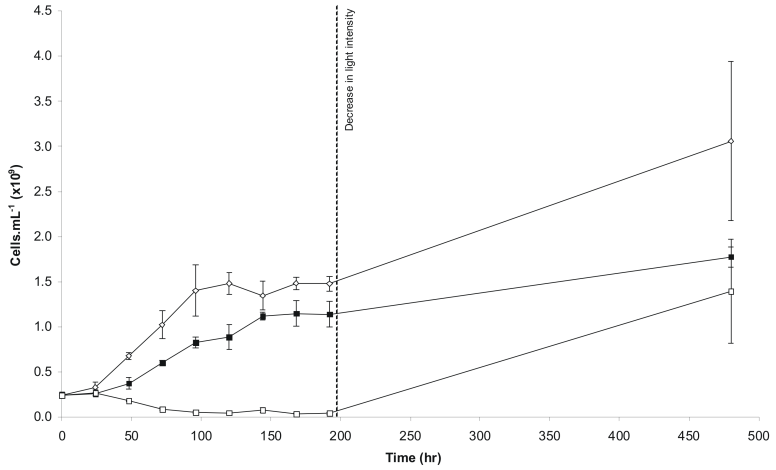

Figure 4.7: The effect of 4.2 µM (open squares) and 42 µM (filled squares) exogenous BMAA on the growth of *Synechocystis* PCC6803 measured over 20 days. Negative control (open diamonds). The reduction in light intensity from 16 to 10 µmol m^{-2} s^{-1} is indicated by a dashed line at 192 h. Error bars denote the standard deviation of the mean (n=3).

4.4 DISCUSSION

The concentration-dependent increase in total free cellular BMAA in cultures grown in the presence of exogenous BMAA suggests that exogenous BMAA is rapidly taken up by *Synechocystis* PCC6803. A decrease in free cellular BMAA following a substantial and rapid initial increase in the free BMAA fraction could suggest that internalised BMAA is metabolised, covalently modified or incorporated into protein. However, no increase in the protein-associated BMAA fraction was observed over 96 h, despite relatively steady elevated free cellular BMAA levels. The relationship between exogenous BMAA and intracellular protein-associated BMAA with time (Figure 4.2) revealed an initial increase in protein-associated BMAA within 10 min of exposure to exogenous BMAA, with significant increases seen only in the presence of 0.5 and 5 µM BMAA and not in the presence of 0.05 µM BMAA. This initial increase was followed by a decrease in and ultimately a complete disappearance of protein-associated BMAA from the cells. This decrease occurred quicker in cells exposed to lower concentrations of BMAA. These data suggest that extracellular BMAA transiently associates with protein following internalisation, but that protein associated BMAA is rapidly modified or metabolised.

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In addition, these data also indicate that free cellular BMAA is not incorporated into proteins in its native form and that internalised BMAA is either metabolised or covalently modified, either in its free form or following incorporation into or association with proteins.

The retardation or inhibition of growth and induction of chlorosis in the presence of exogenous BMAA and the subsequent recovery of growth and photosynthetic pigments under lower light conditions indicate that the negative effect of exogenous BMAA on the growth of *Synechocystis* PCC6803 is both concentration and light dependent. The data from this study suggest that BMAA may induce cellular states that result in chlorosis, as evidenced by the substantial decrease in pigmentation seen in the presence of higher (>4.2 µM) concentrations of exogenous BMAA at a light intensity of 16 µmol m^{-2} s^{-1}. Macronutrient deprivation in cyanobacteria results in a decrease in anabolic processes and an increase in catabolism and generally results in growth arrest (Schwarz and Forchhammer, 2005). More specifically, cellular nitrogen limitation in cyanobacteria results in the induction of a chlorotic state, due to the breakdown of photosynthetic pigments (Sauer *et al.,* 2001; Schwarz and Forchhammer, 2005). The effects of exogenous BMAA on *Synechocystis* PCC6803 therefore mimic cellular states that, among others, would be expected in a population under macronutrient limitation or photoinhibition due to high irradiance. However, further studies are required to establish the links between BMAA, loss of pigmentation and growth arrest.

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As detailed in chapters three and four, BMAA is produced under nitrogen limiting conditions and free cellular BMAA rapidly disappears on the addition of nitrogen to a nitrogen-depleted culture. Furthermore, in *Synechocystis* PCC 6806 BMAA induces a cellular response that resembles quiescence and/or physiological responses characteristic of nitrogen starvation, and consequent chlorosis. These data are highly suggestive that in cyanobacteria the metabolism of BMAA is closely associated with the regulation of cellular nitrogen status. We therefore investigated the role of BMAA as a possible signal molecule by studying the effects that BMAA has on the regulation of the expression of genes that function in the control of nitrogen metabolism in cyanobacteria.

CHAPTER 5: β-*N*-METHYLAMINO-L-ALANINE AS A NITROGEN-CONTROLLED GENE REGULATOR IN CYANOBACTERIA

5.1 INTRODUCTION

Cyanobacteria respond to nutrient limitations by general or specific acclimation processes, and responses to starvation conditions may vary in different cyanobacterial strains. General starvation responses often involve changes to normal metabolism, with increases in catabolism and decreases in anabolic reactions. Cells enter quiescence, a highly regulated low energy metabolic state that is not the same as that which occurs in dormant bacterial spores (Nyström, 2004; Schwarz and Forchhammer, 2005). Quiescence was described by Nyström (2004) as the cessation of growth and reproduction of a bacterial population in response to the depletion of a required nutrient. General acclimation responses to nutrient limitation also typically include structural changes that are a consequence of the accumulation of storage molecules like glycogen, and the breakdown of subcellular structures and intracellular membranes including thylakoid membranes.

5.1.1 Chlorosis – A general acclimation response

Under nutrient limiting conditions it is important for the cell to maintain a balance between the excitation rate (amount of energy produced) and the cellular metabolic rate. In order to maintain such a balance, cells reduce photosynthetic energy output by degrading phycobiliproteins and chlorophyll *a,* photosynthetic pigments that form part of the major light-harvesting complex in cyanobacteria. Also, the degradation of the phycobilisome, and more specifically phycocyanin, releases stored nitrogen that can be used in protein synthesis during acclimation to a new nitrogen status. Due to the loss of pigments, cells under nutrient stress become chlorotic or bleached (Allen and Smith, 1969) a response that is further modulated by factors that include the nutrient that is limited, temperature, carbon dioxide availability and light intensity (Barker-Astrom *et al*., 2005; Collier & Grossman, 1994; Görl *et al*., 1998). In cyanobacteria, both phosphorous and nitrogen starvation induce the most pronounced chlorotic response compared to

other nutrient limitations, however, nitrogen starvation results in the most rapid onset of chlorosis, characterised by complete degradation of the phycobilisomes. Cell bleaching is reversible and following the supply of the limiting nutrient(s), cells return to a vegetative state relatively rapidly. This is possible because during chlorosis cells maintain a very low metabolic activity, 0-1% that of normal actively growing cells (Sauer *et al.,* 2001).

A reduction in phycobilisome content results from the degradation of phycobilisome proteins and the repression of phycobilisome protein synthesis (reviewed in Schwarz and Forchhammer, 2005). These processes are tightly regulated and controlled by environmental triggers and the physiological and biochemical consequences of these triggers. The *nbl* pathway controls pigment degradation and various components of this pathway have been identified (reviewed in Schwarz and Forchhammer, 2005). The expression of NblA (Collier and Grossman, 1994), the main regulator of phycobilisome degradation, is upregulated under general nutrient starvation by other *nbl* regulatory components, NblR, NblS and NblC (reviewed in Schwarz and Forchhammer, 2005). NblR functions in modulating the activities of light harvesting complexes and photosynthesis in response to environmental triggers. Its response is general to all nutrient limitations and is critical for cell survival under such conditions (Schwarz and Grossman, 1998). Interestingly, the signal that triggers this *nbl* pathway is not yet known, but preliminary data suggest that changes in light and redox state may serve as a trigger (van Waasbergen *et al.,* 2002).

Exogenous BMAA induced a chlorotic response in *Synechocystis* PCC6803, characteristic of the chlorosis observed under nitrogen starvation (see chapter 3). As only an increase in the percentage chlorotic cells was observed, with no net decrease in actual cell numbers, the data strongly suggest that the cells entered into quiescence. Also, chlorosis induced by BMAA was reversible, as demonstrated by the complete recovery of pigmentation and growth of the culture. The overall response of *Synechocystis* PCC6803 to BMAA was very similar to a general response to nutrient limitation. This suggested that BMAA, or a BMAA metabolite, may function in the modulation of nutrient stress acclimation responses, acting specifically as a signal for nitrogen deprivation.

5.1.2 Acclimation to nitrogen limitation

Two general response regulators for nitrogen limitation have been identified, the signal transduction protein PII and the DNA binding protein NtcA. Although first thought to be subordinate in nitrogen regulation and global nitrogen control (Lee *et al.,* 2000), PII actually functions synergistically with NtcA and is intimately involved in nitrogen limitation responses in cyanobacteria. This is evidenced by the decrease in RubisCO gene expression in an NtcA/PII dependent manner, under nitrogen starvation conditions (Schwarz and Forchhammer, 2005).

While PII functions in modulating the activity of target proteins, NtcA functions in enhancing or suppressing the transcription of nitrogen controlled regulators. The activities of NtcA and PII are regulated by α-ketoglutarate (2-oxoglutarate), a metabolite that is consumed by the glutamine synthetase – glutamine oxoglutarate amidotransferase (GS-GOGAT) pathway during the synthesis of glutamate via transamination by glutamine (Flores and Herrero, 1994). Due to its direct involvement in ammonium assimilation, the level of cellular α-ketoglutarate is directly indicative of the cellular nitrogen status, where a decrease or absence in ammonia results in an accumulation of α-ketoglutarate and the activation of acclimation responses (Muro-Pastor *et al.,* 2001; Sauer *et al.,* 1999). The nitrogen response regulator, PII, is activated by phosphorylation following cooperative binding to adenosine triphosphate (ATP) and α-ketoglutarate (Forchhammer, 2004). The DNA binding and transcriptional activity of NtcA is stimulated by α-ketoglutarate and repressed under excess nitrogen (Vazquez-Bermudez *et al.,* 2003). NtcA is also self-regulating in that an increase in activated NtcA stimulates an increase in *ntc*A gene expression. There is thus a substantial increase in the cellular levels of NtcA under nitrogen-limited conditions. NtcA and PII also function in a positive feedback loop. Under nitrogen starvation, activated NtcA enhances the expression of the PII gene, *glnB,* as well as PII phosphorylation via the activation of a protein kinase, while phosphorylated PII activates NtcA. The activation of NtcA by phosphorylated PII only occurs in the absence of nitrate, which means that the cell is able to distinguish between nitrate and general nitrogen depletion, by a mechanism that is not yet understood (Schwarz and Forchhammer, 2005).

In addition to NtcA, PII and the *nbl* pathway response regulators, various σ factors also play a role in the regulation of cellular responses to nitrogen limitation. In cyanobacteria there are three groups of σ factors; group one, containing SigA, that modulates the expression of housekeeping genes necessary to maintain cell viability, group 2, containing SigB, SigC, SigD and SigE, are essential to growth under normal cellular conditions, and group 3, consisting of SigF, SigG, SigH and SigI, the functions of which are not yet fully understood in cyanobacteria (Imamura and Asayama, 2009). However, data suggest that in cyanobacteria, σ factors from all three groups function in the expression of nitrogen regulated genes (Imamura and Asayama, 2009). For the purpose of simplicity, the focus of this chapter will be on those σ factors that function in nitrogen starvation acclimation.

The expression of SigA is constant and independent of growth phase under normal physiological conditions. Its expression is also independent of light/dark cycle and of high/low light (Imamura *et al.,* 2003a,b) but expression is decreased by other environmental conditions such as heat shock, salt stress (Imamura *et al.,* 2003a) and nitrogen deprivation (Imamura *et al.,* 2006). The expression of group 2 σ factor, SigB, is up regulated in response to heat shock under mid-exponential phase (Imamura *et al.,* 2003a). It also contributes to the expression of *gln*B (PII protein), regulated by NtcA, under nitrogen-limited conditions during exponential growth. The expression of other NtcA regulated genes, such as *gln*A, is also enhanced by SigB and the levels of this σ factor increase 2-fold under nitrogen starvation, irrespective of growth phase (Imamura *et al.,* 2006). In contract to SigB, SigC functions in the transcription of *gln*B under nitrogen-limited conditions in stationary phase, but not in exponential growth phase (Asayama *et al.,* 2004). SigC therefore plays a central role in stationary-specific gene expression during acclimation to nitrogen deprivation. Inexplicably, the cellular concentration of SigC in stationary phase appears to be half of what it is in exponential growth phase, and remains almost constant irrespective of cellular nitrogen depletion (Imamura *et al.*, 2003a; Imamura *et al.,* 2006). How this σ factor is then specifically activated during stationary phase, or whether all SigC RNA polymerase transcribed genes have additional regulatory elements, remains unknown. SigD is recognised as a nitrogen responsive σ factor and affects the transcript levels of nitrogen regulated genes,

including, *gln*B and *gln*N. Under normal physiological conditions it also functions in controlling the expression of genes that code for proteins that play a role in sugar metabolism and in light responses.

Sigma factors that function in nitrogen deprivation acclimation responses function in a coordinated manner under specific growth conditions, and their expression and activity is very closely regulated by environmental circumstances including laboratory settings such as sampling times or experimental conditions. Additionally, σ factors may effect the transcription of each other either directly at a transcriptional level or indirectly via the interaction with other transcription-regulating proteins. This means that a disruption to one σ factor my impact on the expression and function of another (Imamura and Asayama, 2009).

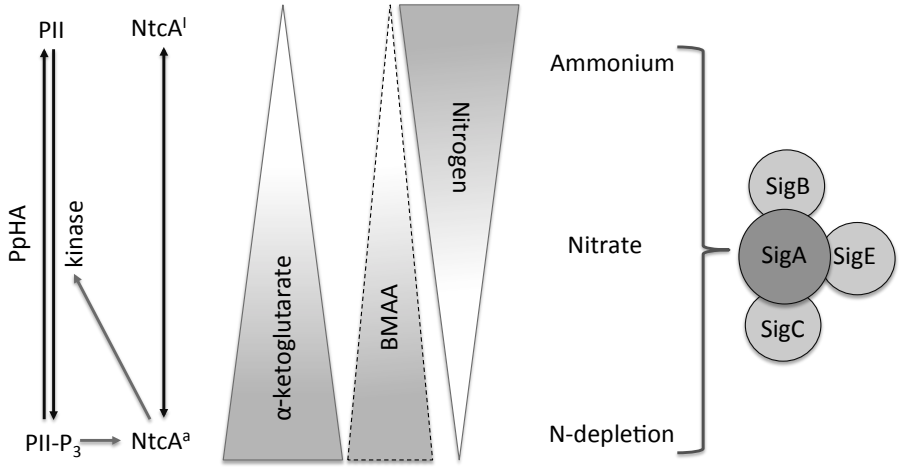

Figure 5.1: A schematic summary of the cellular responses that are controlled by the cellular nitrogen status. The global nitrogen response regulator NtcA and signal transduction protein PII are both inactive (NtcA^{I}, PII, respectively) in the presence of excess nitrogen and low α-ketoglutarate levels. Activated PII (PII-P$_3$) and activated NtcA (NtcA$_a$) function in a positive feedback loop stimulating the activation of each other under nitrogen deprivation. Dephosphorylation and inactivation of PII-P is catalysed by protein phosphatase (PpHA). Sigma factors, SigA, SigB, SigC and SigE, also function in modulating the expression of nitrogen controlled genes. BMAA is produced under nitrogen-limited conditions and disappears from the cell upon addition of ammonia (chapter 3). Adapted from Schwarz and Forchhammer, 2005 and Imamura and Asayama, 2009).

5.1.3 BMAA and gene expression

Recently the effect of BMAA on the expression of nitrogen-controlled genes was investigated (van Zyl, 2013). In *Synechocystis* PCC 6803 cells in stationary phase, exposure to exogenous BMAA affected the transcript levels of selected genes, *sig*A, *nbl*A, *nblS* and *ntc*A, relative to *sig*C expression, which remained constant. Based on these data the author suggested that BMAA might function in the regulation of nitrogen metabolism at a gene expression level (Figure 5.1).

The activities of nitrogen response regulators vary significantly under different growth phases, and whether the effect BMAA has on nitrogen-controlled gene expression is independent of growth phase was unknown. The aim of this study was therefore to investigate the effect of BMAA on gene expression, in exponential growth phase, of the same genes studied by van Zyl (2013).

5.2 MATERIALS AND METHODS

5.2.1 Cultures and growth conditions

An axenic *Synechocystis* PCC6803 culture was maintained in BG11 (Rippka, 1988) at a constant temperature of 23 ± 1 °C under a constant day:night cycle of 14:12 hours. Culture purity was confirmed microscopically during culture maintenance and prior to experimentation. An aliquot of this culture was sub-cultured over a period of three months, maintaining exponential growth and an excess of combined nitrogen in the culture medium by regular batch feeding. The affect of BMAA on gene expression was analysed during exponential growth phase.

5.2.2 Analysis of the effect of BMAA on gene expression

BMAA exposure

A *Synechocystis* PCC6803 culture in exponential growth phase (culture density of OD_{750nm} = ~ 0.400) was aliquoted (300mL) into ten replicate flasks (five control exposures and five experimental exposures). Cultures were acclimatised to individual flasks for 5 hours. BMAA was added to 5 experimental flasks at a final concentration of 100 µM, while only BMAA diluent was added to control flasks. Cultures were incubated under the same conditions as previously described. Samples (100 mL) were taken at 1, 8, and 16 hours following BMAA exposure. Cells were harvested from 100 mL of culture by centrifugation at 5000 x *g* for 10 min. Cell pellets were immediately re-suspended in 1 mL TRIzol® reagent, transferred to a screw-cap ribonucleic acid (RNA) isolation tube, snap-frozen in liquid nitrogen and stored at -80 °C until further processing.

RNA isolation

Cell pellets, re-suspended in TRIzol® reagent, were homogenised with a stainless steel homogenisation bead using a Retsch™ M301 bead homogeniser, at a frequency of 30 ($1\ s^{-1}$) for 5 min twice in succession. Following homogenisation, tubes were centrifuged at 12 000 x *g* for 15 min and the aqueous phase was transferred to a sterile nuclease-free tube. Isopropanol (100%) (0.5 mL) was added to each tube and incubated at room temperature for 10 min. Following incubation, tubes were centrifuged at 12 000 x *g* for 10 min at 4 °C, the supernatant discarded and the pellet re-suspended in 75% ethanol by light vortexing. Ethanol suspensions were centrifuged at 7500 x *g* for 4 min at 4 °C and the supernatant discarded. Pellets were air dried at room temperature for 10 - 15 min. Dried RNA pellets were re-suspended in 20 µL nuclease-free water and incubated at 55 - 60 °C for 10 min. RNA clean-up was performed using a ThermoScientific RNase-free DNase I kit according to the manufacturer's protocol, in which 1 µg RNA was treated with a maximum of 1 U of DNase I (deoxyribonuclease). RNA was stored at –80 °C until further use.

Quantification of gene expression

RNA was reverse transcribed to complementary deoxyribonucleic acid (cDNA) using a BioRad iScript RT kit, according to the manufacturer's protocol. Concentrations of RNA and cDNA were determined using a ThermoScientific NanoDrop 2000c spectrophotometer. cDNA was stored at -20 °C until further use.

Gene transcripts of selected genes (transcription factor *ntc*A, phycobiliprotein degradation *nbl* pathway gene *nbl*A and sigma factor *sig*C) were quantified by means of real time-PCR (RT-PCR) analysis using SsoFast™ EvaGreen® Supermix according to the manufacturer's instructions. The primer sequences for the amplification of these genes were as follows: *ntc*A, forward primer sequence, 5'-ATACTCGAGATGGATCAGTCCCTAACC-3', and reverse primer sequence 5'-TCACTCGAGGGCACTGGTCATAGAGG-3' (Alfonso *et al.,* 2001); *nbl*A, forward primer sequence, 5'-CTTGTGTAACAATTCAGAAAGAC-3', and reverse primer sequence, 5'-CTTCGCGGGCAAGTCTTGCACACG-3' (Luque *et al.,* 2001); *sig*C, forward primer sequence, 5'-AAGCATGCAGAAACCAGCAACGATGAGCC-3', and reverse primer sequence, 5'-CTGGATCCACCCAAATTTTCGTAATAGTCC-3' (Imamura *et al.,* 2003a).

To account for instrument variations, all PCR reactions were performed in duplicate. Each PCR run included a non-template control (NTC), a reaction that did not contain cDNA. RT-PCR reactions (20 µL) were prepared on ice and individual reactions were performed for a specific gene (one primer set per reaction). Reactions contained the following; SsoFast™ EvaGreen® Supermix (10 µL), forward and reverse primer (1 µL each at a final concentration of 500 nM), total template cDNA (1 µg) and RT-PCR-grade RNase/DNase-free H_2O). The RT-PCR protocol was as follows: 1 cycle of 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; 95 °C for 30 s. A melting profile was determined by raising the temperature by 1 °C every 30 s from 55 °C to 95 °C. Each gene was amplified in duplicate.

The efficiencies of RT-PCR reactions were analysed using the $\Delta\Delta C_T$ method of relative quantification, as described in Critical Factors for Successful Real-Time PCR (Qiagen®). In this method the threshold cycle (C_T) values of the control samples (calibrator) are directly compared to that of the experimental samples (sample) based on differences between each sample's C_T values for the target gene (i.e. an affected gene) and the reference gene (i.e. an unaffected housekeeping gene). The method calculates the ΔC_T value for each sample from which the $\Delta\Delta C_T$ value can be calculated. Equations are given below.

ΔC_T *(sample)* $= C_T$ *(target gene)* $- C_T$ *(reference gene)*
ΔC_T *(calibrator)* $= C_T$ *(target gene)* $- C_T$ *(reference gene)*
$\Delta\Delta C_T$ *(sample)* $= \Delta C_T$ *(sample)* $- \Delta C_T$ *(calibrator)*

Given comparable RT-PCR efficiencies of both the target gene and the reference gene the normalised level of target gene expression was calculated as follows:
normalised target gene expression level in sample $= 2^{-\Delta\Delta_T}$.

5.3 RESULTS AND DISCUSSION

BMAA had no significant effect on the transcript levels of the nitrogen-controlled gene, *ntc*A or sigma factor SigC, in *Synchocystis* PCC6803 during exponential growth phase. Expression of SigC remained constant and was used as a reference gene in the normalisation of results. The sensor kinase regulatory gene, *nbl*S, was the only gene that had higher transcript levels relative to SigC. However, the transcript levels of *nbl*S were not notably greater in BMAA exposed cultures compared to control cultures. Data therefore suggest that BMAA did not have an effect on the expression of any of nitrogen-controlled genes tested. However, as the regulatory activities of SigC are repressed in exponential phase (reviewed in Imamura *et al.,* 2009), the absence of a BMAA-induced effect on nitrogen controlled gene expression in stationary phase could suggested that the effect of BMAA on gene expression may be regulated by SigC or SigC controlled response regulators.

The expression of *nbl*A was not analysed in the current study as van Zyl (2013) observed that BMAA did not have a significant effect on *nbl*A expression in *Synechocystis* PCC6803 in stationary phase. Van Zyl (2013) did observe a marked increase in *nbl*S transcription. Under nutrient replete conditions NblS functions in repressing the activity of the phycobilisome degradation regulator, NblA. This repression is stopped during nutrient limitation, enabling NblA to modulate acclimation responses. Considering this, expression of *nbl*S under nutrient replete conditions, as observed in the current study and by van Zyl (2013), is to be expected. However, the BMAA-induced increase in *nbl*S expression and decrease in *nbl*A expression described by van Zyl (2013) does not correspond to previous observations of BMAA induced chlorosis. As the induction of chlorosis is primarily modulated by *nbl*A, how BMAA can induce a decrease *nbl*A transcription and an increase the NblA repressor, NblS, and induce chlorosis is not yet understood. The inconsistencies of these data highlight the complexity of nitrogen controlled regulatory pathways and warrant further investigation of the effect of BMAA on such pathways.

The global nitrogen response regulator NtcA is up regulated in the presence of BMAA, in *Synechocystis* PCC6803 in stationary phase in the presence of nitrogen (van Zyl, 2013). However, data from this study show that during exponential growth phase, BMAA had no effect on the expression of this regulator. This suggests that the effect of BMAA on *ntc*A transcription is growth phase dependent. However, as the actual nitrogen content of the medium was not quantified in either study, the influence of cellular nitrogen status on these BMAA-induced effects cannot be excluded. The enhanced expression of a response regulator under a specific set of cellular conditions does not directly result in the initiation of a regulatory response, as it requires the coordinated function of many other regulatory factors that may not be active or present within the cell under those conditions. Therefore, the effect of BMAA on the expression of *ntc*A may only result in the induction of a nitrogen controlled regulatory response such as chlorosis under very specific cellular conditions.

5.4 CONCLUSION

BMAA has an effect on the expression of the global nitrogen response regulator, NtcA, but this effect is growth phase dependent and absent during exponential growth. Furthermore, data suggest that the expression of certain nitrogen-controlled response regulators that function under nitrogen limiting conditions is affected by BMAA in the presence of nitrogen. It is clear that although it appears that BMAA does play a role in the regulation or response to nitrogen deprivation, the role is complex and further investigations based on these preliminary data are warranted. Work on this topic is ongoing.

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Having established that BMAA metabolism in cyanobacteria is closely associated with nitrogen metabolism, isotopically labelled BMAA had to be chemically synthesised for the use in further BMAA metabolic studies.

CHAPTER 6: CHEMICAL SYNTHESIS OF STABLE-ISOTOPE LABELLED β-*N*-METHYLAMINO-L-ALANINE, L-BMAA-(AMINE-^{15}N)

6.1 INTRODUCTION

Following the discovery of BMAA in *C. circinalis* in 1967 (Vega and Bell) this amino acid has been successfully chemically synthesised for the use in metabolic and toxicity studies (Vega *et al.,* 1968; Reece and Nunn, 1988; Nunn and O'Brien, 1989; Nunn and Ponnusamy, 2009). However, there are few published methods on the chemical synthesis of BMAA, and many of those that are available describe the synthesis of BMAA derivatives (Arnold *et al.,* 1985; Radhakrishna *et al.,* 1979; Ratemi and Vederas, 1994).

In order to further the investigation of BMAA biosynthesis and metabolism in cyanobacteria it was necessary to synthesise BMAA that contained only a single isotope label that could be used in conjunction with isotopically labelled BMAA, L-BMAA-4,4,4-d_3,$^{15}N_2$, containing 5 isotope labels, purchased from the Institute of Ethnomedicine (Wyoming, United States of America).

This chapter will outline the two methods that were used in the chemical synthesis of single isotope labelled L-BMAA. The first method used here to synthesise 2-amino-3-(methyl-[^{15}N]amino)propanoic acid, was published by Hu and Ziffer (1990) and is very similar to the original method used in the synthesis of BMAA by Vega *et al.* (1968). In the Hu and Ziffer (1990) method aqueous [^{15}N]-methylamine-HCl is used as starting substrate and is reacted with 2-acetamidoprop-2-enioc acid (α-acetaminoacrylic acid) to produce 2-acetamido-3-(methyl-[^{15}N]amino)propanoic acid. This derivative is thereafter deacetylated, catalysed by L-amino acylase, to yield 2-amino-3-(methyl-[^{15}N]amino)propanoic acid (Figure 6.1).

A second method was also employed in the synthesis of 2-[^{15}N]amino-3-(methylamino)propanoic acid or L-BMAA-(amine-^{15}N). It is, however, a proprietary method developed by the Institute for Ethnomedicine in Wyoming (United States of America), and unfortunately no details of this method can be disclosed.

6.2 MATERIALS AND METHOD

6.2.1 Method I: As published by Hu and Ziffer (1990)

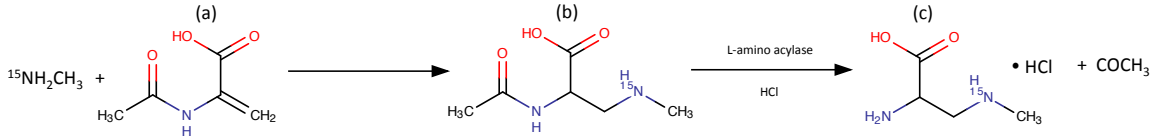

Figure 6.1: A schematic summary of method I (Hu and Ziffer, 1990) used in the synthesis of 2-amino-3-(methyl-[^{15}N]amino)propanoic acid. Aqueous [^{15}N]-methylamine ($^{15}NH_2CH^3$) is reacted with 2-acetamidoprop-2-enioc acid (a) to yield 2-acetamido-3-(methyl-^{15}N-amino)propanoic acid (b) which is deacetylated via reaction with L-amino acylase to yield L-α-amino-β-methyl-[^{15}N]aminopropionic acid hydrochloride (c).

An aqueous [^{15}N]-methylamine solution was prepared by dissolving a frozen [^{15}N]-methylamine-HCL solution (2g) in water (8 mL) with solid NaOH (1.76 g) that was slowly warmed to room temperature. The aqueous [^{15}N]-methylamine solution was distilled in vacuo. The distillate was treated with 2-acetamidoprop-2-enioc acid at 40 °C for 22 h. Unreacted [^{15}N]-methylamine was removed by means of vacuum distillation and the residue was crystallised from ethanol to yield 2-acetamido-3-(methyl-[^{15}N]amino)propanoic acid. In order to increase the yield, unreacted aqueous [^{15}N]-methylamine was again reacted with 2-acetamidoprop-2-enioc acid to yield 2-acetamido-3-(methyl-[^{15}N]amino)propanoic acid as previously described. Reaction of 2-acetamidoprop-2-enioc acid with unreacted [^{15}N]-methylamine was repeated a third time, and the product, 2-acetamido-3-(methyl-[^{15}N]amino)propanoic acid, was pooled.

An aqueous solution of 2-acetamido-3-(methyl-[^{15}N]amino)propanoic acid was prepared and the pH adjusted to 7.5 with 2M LiOH. 2-acetamido-3-(methyl-[^{15}N]amino)propanoic was deacetylated by reaction with Acylase I (EC. 3.5.1.14) from porcine kidney (Sigma Aldrich®), at 37 °C for 30 h. Following incubation, the pH of the solution was adjusted to 5.0 with acetic acid, and charcoal was added and the solution was incubated at 4 °C overnight. Charcoal was removed via filtration and the solution was passed through a weak cation exchange resin (Bio-Rad® 70).

Unhydrolysed 2-acetamido-3-(methyl-[^{15}N]amino)propanoic was eluted with water and again treated with Acylase I and passed through the cation exchange resin as previously described. Unhydrolysed *N*-acetyl was removed from the column by elution with water, while the free amino acid was retained on the column. L-[^{15}N]-BMAA (β-*N*-methyl-[^{15}N]amino-L-alanine) was eluted with 0.3 N HCl, the eluent concentrated and the residue crystallised from aqueous ethanol to yield L-α-amino-β-methyl-[^{15}N]aminopropionic acid hydrochloride.

All chemicals unless otherwise specified were purchased from Sigma Aldrich®.

6.2.2 Method II:

A proprietary method developed by the Institute for Ethnomedicine in Jackson Hole, Wyoming. Experimental details cannot be disclosed.

6.3 RESULTS AND DISCUSSION

In amino acid metabolism the primary amino group is normally metabolically active, and for this reason the synthesis of single isotope-labelled L-BMAA was focussed on producing alpha-^{15}N amino labelled L-BMAA.

L-BMAA-(amine-^{15}N) (L-α-[^{15}N]amino-β-methylaminopropionic acid) was successfully synthesised using proprietary method II. The identity and purity of the final product yielded by method II was confirmed using LC-MS analysis, by comparison of the elution profile with that of standard non-labelled L-BMAA and L-BMAA-4,4,4-d_3,$^{15}N_2$. LC-MS analysis was performed using the EZ:faast™ method selected in chapter 2, as described by Esterhuizen-Londt *et al.* (2011). The resulting chromatogram is shown in Figure 6.2. LC-MS analysis confirmed that an adequate yield of L-BMAA-(amine-^{15}N) was obtained. Results of intermediate steps and associated chemical intermediates are not shown due to the proprietary nature of the method.

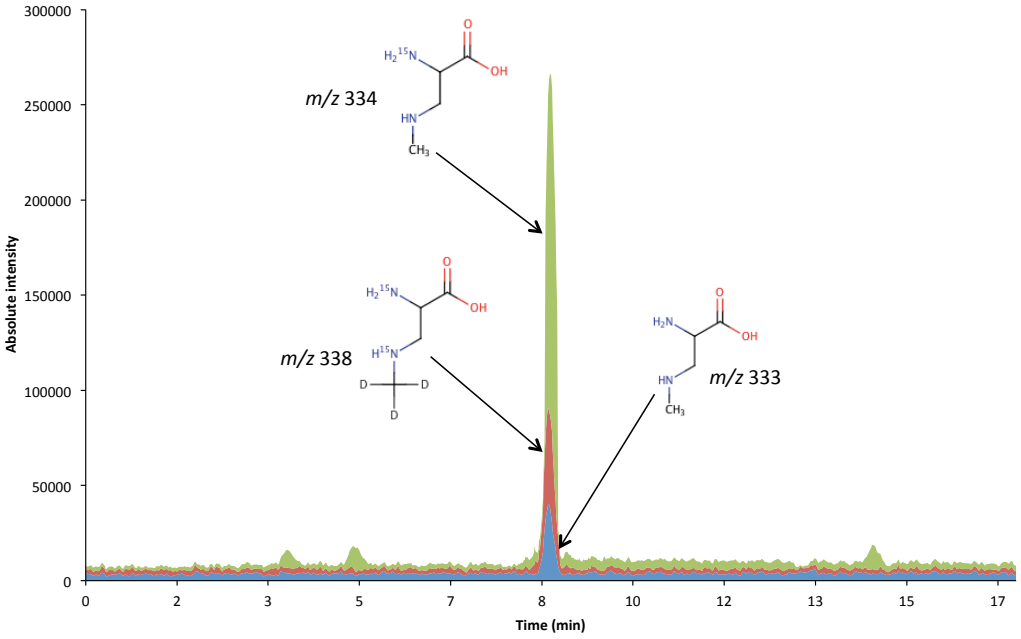

Figure 6.2: LC-MS chromatogram showing the elution profile of propyl chloroformate derivatised chemically synthesised L- α -[^{15}N]amino- β -methylaminopropionic acid (*m/z* 334) compared to the elution profile of propyl chloroformate derivatised L-BMAA-4,4,4-d_3,$^{15}N_2$ (*m/z* 338) (Purchased from the Institute for Ethnomedicine) and commercially available L-BMAA (*m/z* 333) (Sigma Aldrich®).

6.4 CONCLUSION

L-BMAA-(amine-^{15}N) (L-α-[^{15}N]amino-β-methylaminopropionic acid) was successfully synthesised for the use in BMAA metabolism studies.

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As detailed in chapters three, four and five, BMAA metabolism in cyanobacteria appears to be closely linked to nitrogen control. In order to identify specific metabolic pathways that could function in the metabolism of BMAA in cyanobacteria, the transfer of isotope labels from isotopically labelled BMAA to other cellular amino acids was investigated.

CHAPTER 7: β-*N*-METHYLAMINO-L-ALANINE (BMAA) IS A SUBSTRATE FOR GOGAT IN THE CYANOBACTERIUM *SYNECHOCYSTIS* PCC6803

ABSTRACT

The neurotoxic amino acid β-*N*-methylamino-L-alanine is produced by cyanobacteria under nitrogen starvation conditions and its metabolism is closely associated with cellular nitrogen control. Very little is known regarding the metabolism or biosynthesis of this amino acid in the producing organisms and current knowledge is limited to the spontaneous formation of carbamate adducts in the presence of aqueous carbon dioxide, the rapid removal of free cellular BMAA upon the addition of ammonia to nitrogen-starved cyanobacterial cultures, and the link between cellular nitrogen status and BMAA synthesis. Data presented here show that exogenous BMAA is rapidly metabolised by cyanobacteria during which, the primary amino group is rapidly transferred to other cellular amino acids. Furthermore, data suggest that BMAA is metabolised in cyanobacteria via a reversible transamination reaction. This study presents novel data on BMAA metabolism in cyanobacteria and provides the first proposed biosynthetic precursor to BMAA biosynthesis in cyanobacteria.

Keywords: β-*N*-methylamino-L-alanine, BMAA, cyanobacteria, metabolism, biosynthesis

7.1 INTRODUCTION

The cyanobacterial metabolite β-*N*-methylamino-L-alanine (BMAA) is a non-proteinogenic neurotoxic amino acid implicated in the development of neurodegenerative disease. The link between BMAA and cyanobacteria had been argued for many years, a dispute fuelled by the inability of various researchers to detect BMAA in environmental samples and particularly in cyanobacterial cultures (Banack *et al*., 2010).

Submitted for publication: **Downing, S.** and T.G. Downing. β-*N*-methylamino-L-alanine (BMAA) is a substrate for GOGAT in the cyanobacterium *Synechocystis* PCC6803.

The negative correlation between cyanobacterial BMAA production and combined nitrogen availability observed in laboratory cultures (Downing *et al*., 2011) was also observed in environmental samples from a freshwater impoundment (Scott *et. al.,* 2014). These data not only support the BMAA-cyanobacterial link, but also explain in part the absence of BMAA in many cyanobacterial samples.

BMAA is environmentally ubiquitous and is readily taken up by cyanobacteria (Downing *et al.,* 2012) and accumulated by plants (Contardo-Jara *et al.,* 2012; Esterhuizen *et al.,* 2011) and invertebrates and higher animals (Downing *et al*., 2014; Brand *et al*., 2010; Jonasson *et al.,* 2010; Mondo *et al.,* 2012). When BMAA is present in an aquatic environment, it not only bioaccumulates in plants and animals, but also significantly biomagnifies (Banack and Cox, 2003; Cox *et al.,* 2003, Jonasson *et al*., 2010; Brand *et al*., 2010). A recent study showed evidence for the metabolism of BMAA via reversible covalent modification in freshwater mussels (Downing *et al*., 2014). However, information on the metabolism of BMAA in the environment and specifically in cyanobacteria remains extremely limited, with most studies that have investigated BMAA metabolism having done so *in vitro* or in mammalian models.

BMAA spontaneously forms carbamate adducts in the presence of bicarbonate (Weiss and Choi, 1988; Weiss *et al.,* 1989; Nunn and O'Brien, 1989; Mroz *et al.,* 1989) via the reversible reaction of aqueous carbon dioxide with either the primary or secondary amino group to form one of two possible adducts; α-*N*-carboxy- or β-*N*-carboxy-β-*N*-methylamino-L-alanine (BMAA-α-NCO_2 and BMAA-β-NCO_2, respectively) (Myers and Nelson, 1990). Furthermore, Moura (2009) showed that the cyclisation of BMAA carbamate adducts (more specifically BMAA-β-NCO_2) to form 1-methyl-2-oxoimidazolidine-4-carboxylic acid is chemically favourable, occurs spontaneously and is an intermediate in the equilibrium reaction between BMAA and alpha or beta *N*-carboxy-β-*N*-methylamino-L-alanine *in vitro*. *In vitro,* L-BMAA can be oxidised by L-amino acid oxidase, to yield the iminium ion of BMAA, which, following hydrolysis, yields the BMAA keto acid, β-*N*-methylaminopyruvate that in turn can yield *N*-methyl glycine following oxidative decarboxylation (Hashmi and Anders, 1991).

Furthermore, Nunn and Ponnusamy (2009) showed that *in vitro,* BMAA reacts with pyridoxal-5'-phosphate to yield ammonia and methylamine. In rats BMAA is metabolised to yield the acetylated BMAA derivative, α-acetamido-β-methylaminopropionic acid (Reece and Nunn, 1988), and in rat brain slices (Nunn and Ponnusamy, 2009) the presence of BMAA resulted in a decrease in tissue glutamine, taurine, serine and glycine levels and in an increase in alanine and ammonia levels, while in rat tissue homogenates the presence of BMAA resulted in an increase in alanine and ammonia in liver homogenates and in an increase in methylamine in liver and kidney homogenates. The demethylated derivative of BMAA, 2,3-diaminopropanoic acid, was detected in both liver and kidney homogenates (Nunn and Ponnusamy, 2009).

Current published data indicate that BMAA metabolism in cyanobacteria is very closely associated with cellular nitrogen metabolism. Downing *et al*. (2011) showed that BMAA is produced by cyanobacteria under nitrogen limiting conditions and more importantly, that free cellular BMAA disappears rapidly from the cell upon the addition of ammonia to nitrogen deplete cyanobacterial cultures. Moreover, the uptake of exogenous BMAA by cyanobacteria causes chlorosis and a decrease in growth in a concentration dependent manner (Downing *et al*., 2012) mimicking the chlorosis induced by nitrogen deprivation (Allen and Smith, 1969).

With regard to BMAA biosynthetic mechanisms, Brenner *et al*., (2003) suggested a putative two-step pathway for BMAA biosynthesis in cycad plants, which involved the direct amination of a beta-substituted alanine residue with ammonia. Araoz *et al.* (2010) identified putative cyanobacterial genes, orthologous to the cycad genes Brenner *et al.* (2003) based his suggested pathway on, and suggested that the BMAA biosynthetic pathway proposed by Brenner *et al.* (2003) may also function in cyanobacteria. However, Downing *et al*. (2011) argued that new data showed the synthesis of BMAA in cyanobacteria only in the absence of free cellular nitrogen, and the disappearance of BMAA in the presence of excess ammonia, which would make the pathway proposed by Brenner *et al.,* (2003), which relies heavily of the presence of ammonia, implausible.

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Metabolism of all proteinogenic amino acids is closely connected and interlinked and even the synthesis of unusual or non-proteinogenic amino acids, such as 2,4-diaminobutyric acid is closely associated with the synthesis of other cellular amino acids (Nigam and Ressler, 1966; Rajagopal *et al.,* 1969; Steinhauser *et al.,* 2012). In this study the metabolism of BMAA in cyanobacteria was investigated by examining the redistribution of stable isotopes from isotopically labelled BMAA to other proteinogenic and non-proteinogenic amino acids.

7.2 MATERIAL AND METHODS

7.2.1 Strains and culture conditions

An axenic *Synechocystis* PCC6803 (referred to from here on as *Synechocystis*) culture was obtained from the Pasteur Culture Collection (Paris, France). Cultures were maintained in BG_{11} media (Rippka, 1988) under sterile conditions at a temperature of 23°C (±1°C) with constant illumination at a light intensity of 16 µmol m^{-2} sec^{-1} (Triton Dayglo©). Culture purity was regularly monitored microscopically.

7.2.2 Isotopically labelled BMAA and exposure

Isotopically labelled BMAA, L-BMAA-4,4,4-d_3,$^{15}N_2$ (also referred to here as $^{5+}$BMAA) was purchased from the Institute of Ethnomedicine (Jackson, Wyoming) and L-α-[^{15}N]amino-β-methylaminopropionic acid (also referred to here as $^{1+}$BMAA) was synthesised using a proprietary method. Replicate *Synechocystis* cultures were supplemented with either L-BMAA-4,4,4-d_3,$^{15}N_2$ or L-α-[^{15}N]amino-β-methylaminopropionic acid at a final concentration of 100 µM. As the aim of this experiment was to investigate the metabolism of BMAA by cyanobacteria, exposure concentrations exceeded those associated with typical environmental BMAA concentrations. Cyanobacterial cells were collected at 1, 6, 12, 24 and 48 hours following supplementation by centrifugation at 5000 *x g* for 10 min.

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In order to monitor cellular BMAA levels following exposure to L-BMAA-4,4,4-d_3,$^{15}N_2$ but in the absence of continued exposure to L-BMAA-4,4,4-d_3,$^{15}N_2$ a second exposure study was conducted in which *Synechocystis* cultures were exposed to L-BMAA-4,4,4-d_3,$^{15}N_2$ at a final concentration of 100 µM for 1 hour. After exposure, cells were pelleted via centrifugation as described above, washed twice with BMAA-free culture medium and re-suspended in BMAA-free medium to the same cell density as during exposure. Cell samples were collected at 1, 6, 12, 24 and 48 hours following transfer to BMAA-free medium, as described above. All cell pellet samples where snap frozen in liquid nitrogen directly after sampling, and stored at -80 °C until further analysis.

7.2.3 Inhibition of GS-GOGAT enzymes

In order to identify possible routes for the re-distribution of labels from L-BMAA-4,4,4-d_3,$^{15}N_2$, distribution patterns in glutamate and glutamine were analysed in the presence of methionine sulfoximine (MSX), an inhibitor of glutamine synthetase (GS) and the glutamine analogue, 6-diazo-5-oxo-L-norleucine (DON), an inhibitor of glutamine oxoglutarate amidotransferase (GOGAT). *Synechocystis* was exposed to each enzyme inhibitor separately, at a final concentration of 1 mM for 2 h prior to the addition of 100 µM L-BMAA-4,4,4-d_3,$^{15}N_2$. Cultures were exposed to L-BMAA-4,4,4-d_3,$^{15}N_2$ for 24 h, after which they were harvested and stored as previously described.

7.2.4 Extraction and analysis of amino acids

The free cellular amino acids fraction, being the fraction most representative of primary amino acid metabolism, was extracted from cell pellets using trichloroacetic acid (TCA) protein precipitation as described Downing *et al*. (2011). Extracts of free amino acids were derivatised using propyl chloroformate, separated by high performance liquid chromatography (HPLC) and anlaysed using single quadrupole mass spectrometry (MS) as described by Downing *et al.* (2012). Analysis of isotopically labelled amino acids, also referred to as amino acid isotopologues, was based on HPLC retention times and mass

spectrometric detection of amino acid isotopologue parent ions as described by Downing *et al*. (2014). Analysis of the incorporation of labels into proteinogenic and non-proteinogenic amino acids was based on the relative abundance of amino acid isotopologues extracted from L-BMAA-4,4,4-d_3,$^{15}N_2$-exposed cultures, normalised against control cultures. Due to the nature of this study, the aim being to investigate the metabolism of BMAA in cyanobacteria based on the redistribution of labels, no absolute quantification of labelled or unlabelled amino acids was performed. Data represent the relative abundance of labelled amino acids based on LC/MS peak areas, unless otherwise stated.

7.3 RESULTS AND DISCUSSION

7.3.1 Uptake of exogenous isotopically labelled BMAA

As previously reported by Downing *et al.* (2012), extracellular BMAA is rapidly taken up by cyanobacteria. Figure 7.1 (insert) shows the uptake of L-BMAA-4,4,4-d_3,$^{15}N_2$ by *Synechocystis* over 48 hours, with a significant (p<0.05) increase in free cellular labelled BMAA within the first hour of exposure. Intracellular $^{5+}$BMAA levels continued to increase throughout the 48 hours exposure period with the rate of uptake decreasing after the initial 24 hours. In addition to $^{5+}$BMAA, the $^{4+}$BMAA isotopologue was also detected in all samples, and, like $^{5+}$BMAA, was absent from all samples taken prior to the addition of $^{5+}$BMAA. $^{4+}$BMAA was present in detectable but insignificant amounts in the $^{5+}$BMAA stock, and all $^{4+}$BMAA analysis was normalised accordingly. No other BMAA isotopologue was detected in any of the samples exposed to L-BMAA-4,4,4-d_3,$^{15}N_2$. Figure 7.1 shows the change in the ratio of $^{4+}$BMAA to $^{5+}$BMAA in *Synechocystis* continuously exposed to L-BMAA-4,4,4-d_3,$^{15}N_2$ and *Synechocystis* exposed to L-BMAA-4,4,4-d_3,$^{15}N_2$ for 1 h after which the cells were transferred to BMAA free medium. Data (Figure 7.1) suggest that, in the absence of continual exposure to exogenous BMAA, there were fluctuations in the ratio $^{4+}$BMAA to $^{5+}$BMAA, with a significant (p<0.05) increase in this ratio after 6 and 24 hours and at 48 hours relative to t_0, with an overall increasing trend.

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This increase suggests that one stable isotope of $^{5+}$BMAA was replaced with a non-labelled isotope. As discussed below, the replacement of the primary amine group of L-BMAA-4,4,4-d_3,$^{15}N_2$ occurs via a transamination reaction in which the BMAA keto acid, β-*N*-methylaminopyruvate, is re-aminated.

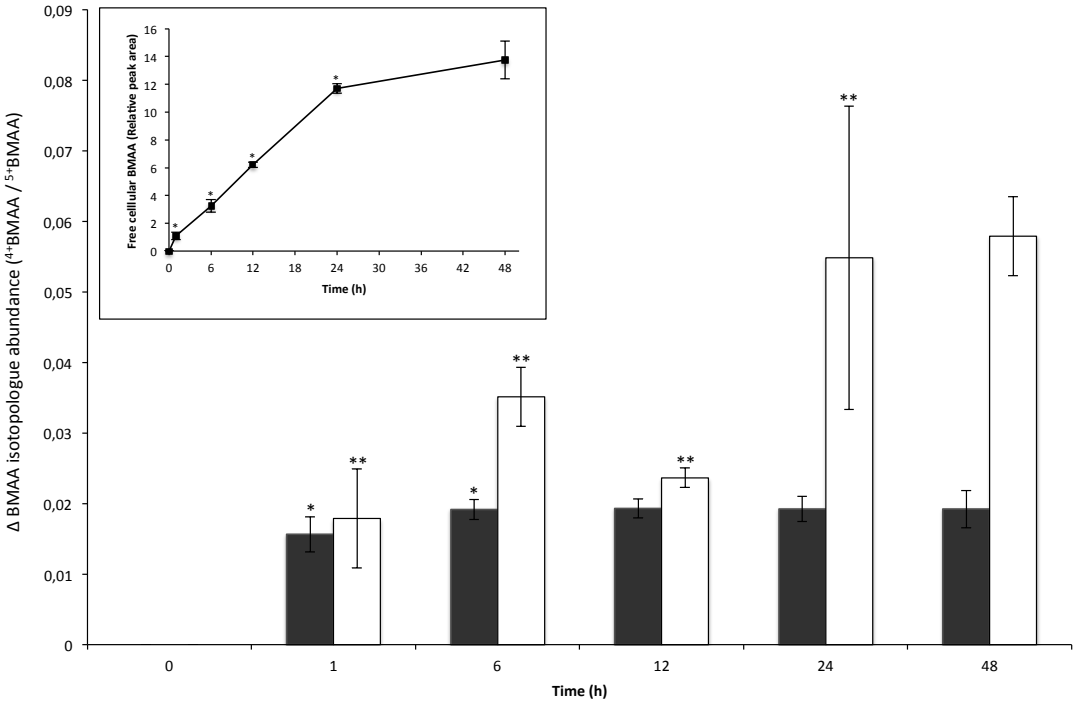

Figure 7.1: The change in the abundance of BMAA isotopologues, depicted as the ratio of $^{4+}$BMAA to $^{5+}$BMAA in *Synechocystis* continuously exposed to 100 µM L-BMAA-4,4,4-d_3,$^{15}N_2$ over 48 hours (grey bars) and in *Synechocystis* exposed to 100 µM L-BMAA-4,4,4-d_3,$^{15}N_2$ for 1 h and thereafter grown in the absence of exogenous L-BMAA-4,4,4-d_3,$^{15}N_2$ (open bars). Error bars denote standard deviations where n=3. Significant ($p < 0.05$) differences from previous time points are indicated by (*) for continuously exposed cultures and by (**) for 1 h exposed cultures. (Insert) Total free cellular BMAA (sum of all isotopologues detected) in *Synechocystis* continuously exposed to 100 µM L-BMAA-4,4,4-d_3,$^{15}N_2$ over 48 hours.

7.3.2 Redistribution of stable isotope labels from labelled BMAA

When analysing the redistribution of BMAA-originating labels throughout the free and protein-associated cellular amino acid pools, changes in the abundance of amino acid isotopologues can often be misleading due to the presence of naturally abundant stable isotopes. All amino acid isotopologue data were normalised to the natural isotope abundance, as determined at t_0 (prior to the addition of labelled BMAA).

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Furthermore, it would be expected that the abundance of isotopologues of any given amino acids would fluctuate in parallel given that the natural abundance of atomic stable isotopes is not altered. Thus, an increase in abundance of $^{1+}$isotopologues due to label incorporation would automatically result in the proportional increase in the corresponding $^{2+}$isotopologue (Figure 7.3). An increase in the ratio of $^{2+}$ to $^{1+}$isotopologues would therefore be indicative of an increase in the abundance of amino acid $^{2+}$isotopologues due to the incorporation of two labels originating from BMAA-4,4,4-d_3,$^{15}N_2$.

The redistribution of L-BMAA-4,4,4-d_3,$^{15}N_2$ labels to selected amino acids (Glu, Gln, Asp, Asn, Ser, Lys, Orn, Sar and Val) was analysed using LC/MS. Relative quantification of amino acids and isotopologues was based on LC/MS peak areas of amino acid parent ions standardised to the internal standard, methionine-d3. Redistribution of labels was defined by a change, relative to control samples, in the abundance of amino acid isotopologue parent ions (*m/z* +*x*), where *x* equals 0 through 5.

Although thorough analysis of the isotopologue abundance of nine selected amino acids listed above was performed, for simplicity, focus here is given to glutamate and glutamine, as these two amino acids are central in amino acid metabolism. Following the continuous exposure of *Synechocystis* to L-BMAA-4,4,4-d_3,$^{15}N_2$, there was a significant (p<0.05) increase in the amount of the glutamate isotopologue, $^{1+}$Glu, within 1 hour of exposure (Figure 7.2, upper pane), which increased steadily over the subsequent 12 hours, after which no significant changes were observed. No substantial significant increase was observed in the abundance of glutamate isotopologue, $^{2+}$Glu. Based on the ratio of $^{2+}$Glu to $^{1+}$Glu (data not shown) the observed slight increase in the abundance of $^{2+}$Glu, corresponding to a relatively large increase in $^{1+}$Glu, was attributed to the presence of naturally abundant isotopologues and the increased $^{1+}$Glu at those time points. The total amount of cellular glutamate decreased gradually over the first 24 hours of exposure, after which it increased again during the last 24 hours of exposure.

A significant (p<0.05) increase in the abundance of the glutamine isotopologue, $^{1+}$Gln, was observed after 12 hours of exposure of *Synechocystis* to L-BMAA-4,4,4-d_3,$^{15}N_2$. Compared to $^{1+}$Glu, the increase in $^{1+}$Gln was relatively delayed, only occurring after 12 hours of exposure, but was greater, with approximately 56 ± 3 % of all cellular glutamine being $^{1+}$Gln. As shown in Figure 7.2, the total amount of cellular glutamine continually increased over the exposure period, indicating that $^{1+}$Gln was *de novo* synthesised. No $^{2+}$Gln or any other glutamine isotopologue was detected at any time point. Data, as depicted in Figure 7.2, therefore strongly suggest that a single stable isotope label was redistributed from L-BMAA-4,4,4-d_3,$^{15}N_2$ to free cellular glutamine and glutamate.

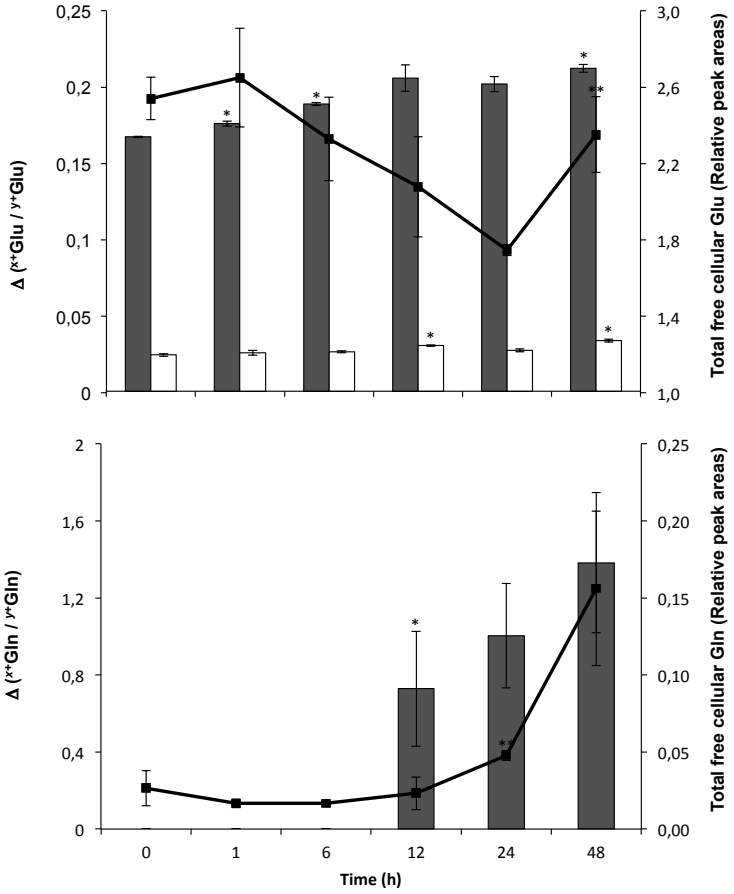

Figure 7.2: The change in the abundance of isotopologues depicted as the ratio of amino acid $^{1+}$isotopologues to native isotopologues (dark grey bars) and the ratio of amino acid $^{2+}$isotopologues to native isotopologues (open bars) in free cellular glutamate (upper pane) and glutamine (lower pane) pools, in *Synechocystis* continuously exposed to 100 µM L-BMAA-4,4,4-d_3,$^{15}N_2$ over 48 h. All graphs depict LC/MS peak areas normalised against internal standard, methionine-d3. The total free cellular amino acid pool of Glu and Gln (upper and lower pane, respectively) are indicated by a solid line. Error bars depict standard deviations where n=3 and significant (p<0.05) differences from previous time points are indicated by an asterisk (*) for isotopologue ratios and by (**) for total free cellular amino acid pools.

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In order to evaluate which isotope label of L-BMAA-4,4,4-d_3,$^{15}N_2$ was being redistributed, the redistribution of L-BMAA-4,4,4-d_3,$^{15}N_2$ labels to glutamate and glutamine was compared to the redistribution pattern of labels in the presence of L-α-[^{15}N]amino-β-methylaminopropionic acid (L-BMAA-(amine-^{15}N). In the presence of L-BMAA-(amine-^{15}N) a similar redistribution of a single isotope label to glutamate and glutamine was observed, confirming that it was the primary amino group that was being transferred. More importantly, this transfer was via a transamination reaction and not via deamination of BMAA to yield free cellular $^{15}NH_4^+$, as evidenced by the lack of $^{2+}$Gln. In cyanobacteria, glutamate/glutamine metabolism involves two sequentially functioning enzymes, glutamine synthetase (GS) and glutamine oxoglutarate amidotransferase (GOGAT). In this GS-GOGAT pathway, glutamine synthetase catalyses the direct amination of glutamate with NH_4^+. Glutamine thus acquires its primary amino group from glutamate and its secondary amide from free cellular NH_4^+. In the presence of $^{1+}$Glu, already shown to be present in the cell after 1 hour of exposure to L-BMAA-4,4,4-d_3,$^{15}N_2$ (Figure 7.2), the generation of $^{15}NH_4^+$ via the deamination of either L-BMAA-4,4,4-d_3,$^{15}N_2$ or L-BMAA-(amine-^{15}N) would therefore result in the production of glutamine-$^{15}N_2$ ($^{2+}$Gln), as both the primary amino group (originating from $^{1+}$Glu) as well as the secondary amide (originating from $^{15}NH_4^+$) would be ^{15}N-labelled. Figure 7.2 shows an increase in $^{1+}$Glu without a concomitant increase in $^{2+}$Gln within the exposure period. As no significant increase in glutamine-$^{15}N_2$ ($^{2+}$Gln) was observed throughout the entire exposure period, BMAA was not deaminated.

As an additional confirmation that BMAA was not deaminated to yield $^{15}NH_4^+$, the relative abundance of asparagine isotopologues was determined. During the synthesis of asparagine, aspartate, synthesised via the transamination of oxaloacetate by glutamate, is transaminated via the secondary amide of glutamine. As discussed above, the deamination of labelled BMAA to yield $^{15}NH_4^+$ would result in glutamine-$^{15}N_2$ and subsequently, given the presence of aspartate-^{15}N (Figure 7.3), in the increase in asparagine-$^{15}N_2$ (H_2-$^{15}NCOCH_2CH(^{15}NH_2)CO_2H$). However, no increase asparagine-$^{15}N_2$ ($^{2+}$Asn) over and above the natural abundance was observed throughout the exposure period, as clearly indicated in Figure 7.3a.

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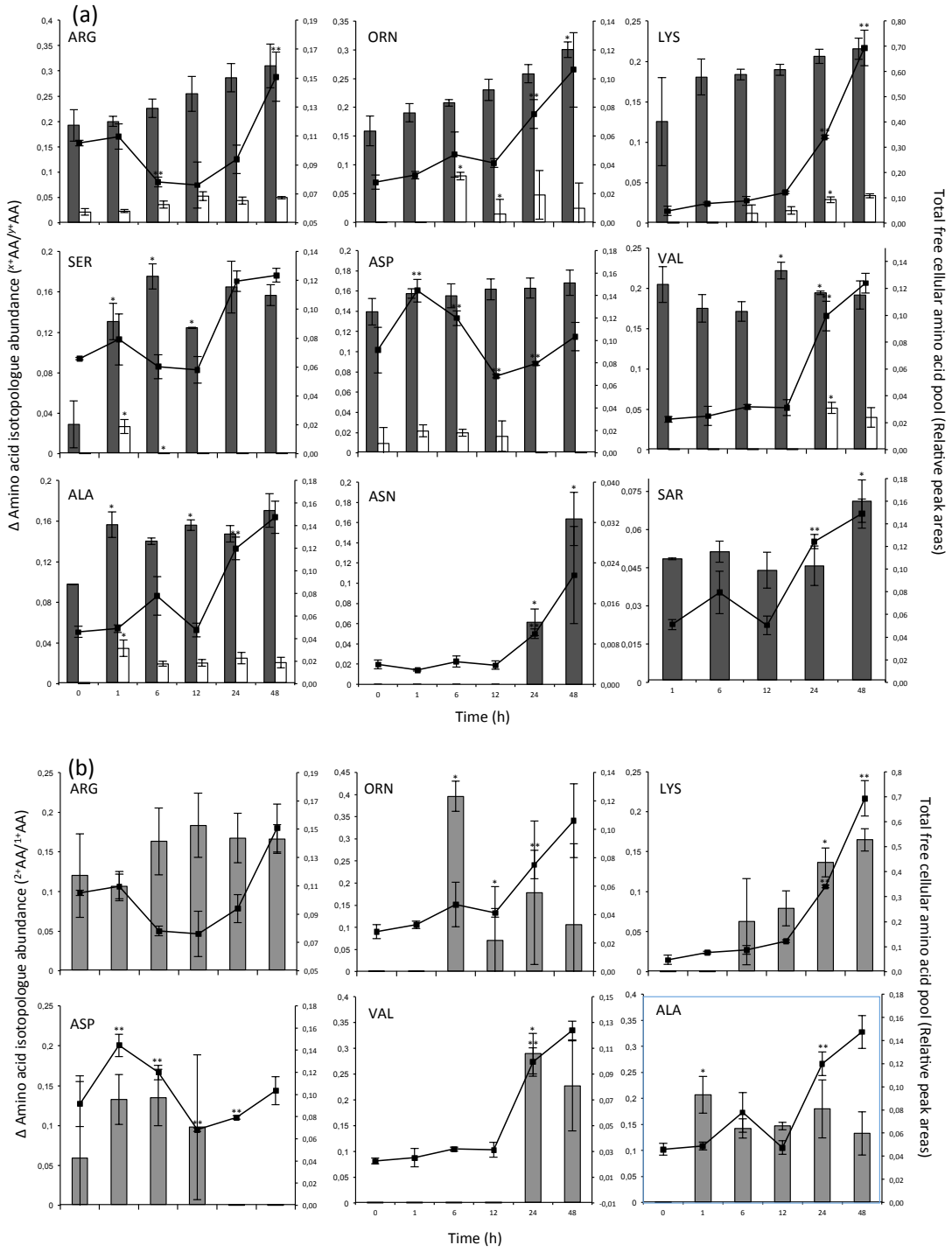

Figure 7.3: (a) The change in amino acid isotopologues in *Synechocystis* continuously exposed to 100 µM L-BMAA-4,4,4-d_3,$^{15}N_2$ over 48 h depicted as the ratio of amino acid $^{1+}$isotopologues to native isotopologues (dark grey bars) and the ratio of amino acid $^{2+}$isotopologues to native isotopologues (light grey bars), only for amino acids in which $^{2+}$isotopologues were detected. (b) The change the ratio of amino acid $^{2+}$isotopologues to $^{1+}$isotopologues, only for amino acids in which $^{2+}$isotopologues were detected. The total free cellular amino acid pool of respective amino acids is indicated by a line in both (a) and (b). All graphs depict LC/MS peak areas normalised against internal standard, methionine-d3. Error bars depict standard deviations where n=3 and significant (p<0.05) differences from previous time points are indicated by an asterisk (*) for isotopologue ratios and by (**) for total free cellular amino acid pools.

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Therefore, analysis of the redistribution patterns of the primary amino group of BMAA reveals that BMAA metabolism in cyanobacteria does not involve a simple deamination reaction but more likely involves transamination with a corresponding keto acid, to produce the BMAA keto acid, β-*N*-methylaminopyruvate.

The redsitribution of L-BMAA-4,4,4-d_3,$^{15}N_2$ labels to cellular amino acids other than Glu and Gln is shown in Figure 7.3. The clear corresponding increases in $^{1+}$ and $^{2+}$isotopologues of various amino acids linked in biosynthesis further confirm that the primary amino group of BMAA is transferred and redistributed. In some cases, such as in valine, increases $^{2+}$isotopologues following 24 or 28 hours exposure to $^{5+}$BMAA suggests redistribution of deuterium atoms due to further catabolism of $^{5+}$BMAA.

7.3.3 The role of GS-GOGAT in the metabolism of BMAA

The labelled BMAA primary amine nitrogen was redistributed to glutamate and glutamine. In the presence of methionine sulfoximine (MSX), a GS inhibitor, the amount of label incorporated into Glu was unaffected, but was significantly reduced in Gln. As MSX inhibits Gln synthesis from Glu, such a decrease would be expected. The fact that there was still some incorporation of label into Gln is due to other biosynthetic pathways such as asparagine metabolism that feed via glutamate back into glutamine.

The most important datum however, is that the inhibition of GOGAT by 6-diazo-5-oxo-L-norleucine (DON) almost completely inhibited the redistribution of BMAA labels to both glutamate and glutamine. This suggests that the metabolism of BMAA and the transamination of its primary amine group are catalysed by GOGAT. This may also explain the accumulation of labelled glutamine in the presence of abundant L-BMAA-4,4,4-d_3,$^{15}N_2$, since BMAA outcompetes glutamine for GOGAT binding at these high concentrations.

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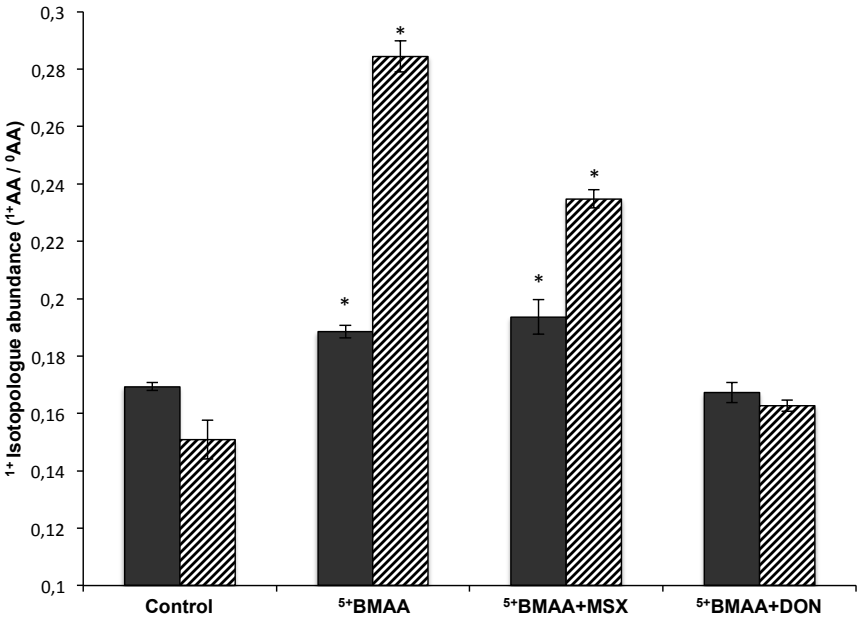

Figure 7.4: The abundance of $^{1+}$isotopologues, depicted as the ratio of $^{1+}$isotopologue abundance to native amino acid (AA) isotopologues of glutamate (solid bars) and glutamine (diagonally striped bars), respectively, in *Synechocystis* exposed to 100 µM L-BMAA-4,4,4-d_3,$^{15}N_2$ for 24 h in the presence of GS inhibitor MSX or GOGAT inhibitor DON. The graph depicts LC/MS peak areas normalised against internal standard, methionine-d3. Error bars depict standard deviations where n=3 and significant (p<0.05) differences from the control (representative of the natural abundance of amino acid isotopologues) are indicated by an asterisk (*).

7.4 CONCLUSION

This study provides the first proposed mechanism of BMAA metabolism in cyanobacteria (Figure 7.5). Data suggest that BMAA is a substrate for the enzyme GOGAT, which catalyses transmination reactions with the primary amine group of BMAA. These data suggest that the last step in BMAA biosynthesis involves the reversible amination of a BMAA-precursor keto acid.

As BMAA metabolism is evidently closely associated with cellular nitrogen balance and the associated cellular responses to nitrogen stress, it is conceivable that the starting substrate in BMAA biosynthesis is generated during cellular nitrogen stress responses, such as protein or nucleotide breakdown, regulated degradation of photosynthetic pigments (phycobiliproteins) or metabolism of nitrogen stores (cyanophycin).

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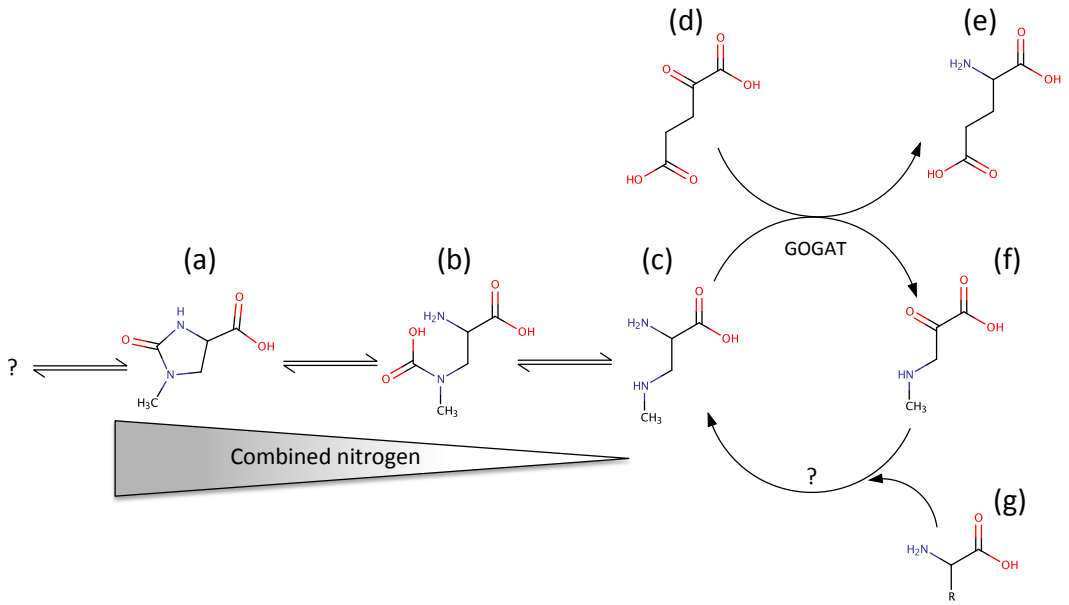

Figure 7.5: A proposed pathway for BMAA metabolism in cyanobacteria. BMAA is in equilibrium between L-BMAA (c) BMAA-β-NCO_2 (b) and cyclic BMAA-β-NCO_2 (a). Under nitrogen-limited conditions L-BMAA (c) is released from a cyclic BMAA-β-NCO_2 derivative (a) and transaminates α-ketoglutarate (d) to produce L-glumatate (e) and the BMAA keto-acid, β-*N*-methylamino pyruvate (f). The BMAA keto-acid is transminated to produce L-BMAA and cyclic BMAA-β-NCO_2 that could be further modified.

Furthermore, considering the spontaneous formation of BMAA-carbamate adducts, the possibility of amination of such carbamates and the evidence for the chemical favorability of cyclic BMAA-carbamate adducts, the role of these BMAA derivatives in the BMAA biosynthetic pathway should not be overlooked.

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In cyanobacteria BMAA is a substrate for GOGAT. Given the chemical reactivity of BMAA under physiological conditions the metabolism of BMAA in other environmentally relevant model organisms seemed likely. It is important to know what happens to the molecule in the environment in order to assess the risk of human BMAA exposure.

CHAPTER 8: β-*N*-METHYLAMINO-L-ALANINE (BMAA) METABOLISM IN THE AQUATIC MACROPHYTE *CERATOPHYLLUM DEMERSUM*

ABSTRACT

The cyanobacterial neurotoxin β-*N*-methylamino-L-alanine (BMAA) bioaccumulates and biomagnifies within the environment. However, most reports on the environmental presence of BMAA focus on the presence of BMAA in animals rather than in plants. Various laboratory studies have reported that this neurotoxin, implicated in neurodegenerative disease, is rapidly taken up by various aquatic and terrestrial plants, including crop plants. In this current study the metabolism of BMAA in the aquatic macrophyte *Ceratophyllum demersum* was investigated using stable-isotope labelled BMAA. Data show that the toxin is rapidly removed from the environment by the plant. However, during depuration cellular BMAA concentrations decrease considerably, without excretion of the toxin back into the environment and without catabolism of BMAA, as evidenced by the absence of label transfer to other amino acids. This strongly suggests that BMAA is metabolised via covalent modification and sequestered inside the plant as a BMAA-derivative. This modification may be reversible in humans following consumption of BMAA-containing plant material. This data therefore impacts on the assessment of the risk of human exposure to this neurotoxin.

Keywords: β-*N*-methylamino-L-alanine, BMAA, aquatic, metabolism, macrophyte

8.1 INTRODUCTION

β-*N*-methylamino-L-alanine (BMAA) is an environmentally ubiquitous neurotoxin that has been implicated in the development of neurodegenerative disease. This unusual non-protein amino acid appears to be present in most cyanobacteria (Cox *et al*., 2005; Esterhuizen and Downing, 2008), and studies, both on a laboratory (Downing *et al*., 2011) and environmental scale (Scott *et al*., 2014), show that biosynthesis of this neurotoxin by cyanobacteria occurs under nitrogen limiting conditions.

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The presence of BMAA in the environment is regularly reported, with bioaccumulation in aquatic invertebrates and vertebrates (Banack and Cox, 2003; Jonasson *et al.,* 2010; Brand *et al*., 2010; Mondo *et al*., 2012). However, limited information is available on the fate and/or persistence of BMAA within the environment, an understanding of which is important for the assessment of the risk of human exposure to this toxin. There is evidence that BMAA can be metabolised or reversibly covalently modified in the environment in selected freshwater mussels (Downing *et al*., 2014). Furthermore, *in vitro* and *in vivo* studies in mammalian models have suggested various possible ways in which BMAA may be metabolised, including acetylation (Reece and Nunn, 1988), oxidation by L-amino acid oxidase followed by hydrolysis and decarboxylation of subsequent metabolites (Hashmi and Anders, 1991), and an *in vitro* reaction with pyridoxyl-5'-phosphate yielding ammonia and methylamine (Nunn and Ponnusamy, 2009). *In vitro* studies on mammalian tissue homogenates showed increases in ammonia and alanine in liver homogenates as well as an increase in methylamine and the presence of 2,3-diaminopropanoic acid, the demethylated BMAA derivative, in liver and kidney homogenates exposed to BMAA (Nunn and Ponnusamy, 2009). However, broader studies on the exact mechanisms of BMAA metabolism in a range of environmentally relevant organisms are lacking.

Although reports on the environmental BMAA content of terrestrial or aquatic macrophytes are limited to selected cycad species, various studies have reported on the uptake and physiological effect of BMAA in other aquatic and terrestrial macrophytes on a laboratory scale. Commonly, BMAA is readily taken up by macrophytes (Esterhuizen *et al.,* 2011; Contardo-Jara *et al*., 2013) is distributed throughout root, stem and leaf tissues (Niyonzima, 2011; Contardo-Jara *et al.,* 2014) and exposure to BMAA may result in morphological defects (Brenner *et al*., 2000) and/or some physiological stress (Contardo-Jara *et al.,* 2013; Esterhuizen-Londt *et al.,* 2011). In all studies, however, focus is given to the uptake and the effects of BMAA on the plants and not to the fate of the compound itself. It is well known that plants employ specific detoxification mechanisms to protect against the accumulation and toxic effects of xenobiotics or other toxic compounds, including cyanobacterial toxins (Pflugmacher *et al.,* 1998).

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Biotransformation systems in plants routinely involve three phases. In phase I, the toxic compound is "activated", reactions during which reactive groups are either exposed or added in order to prepare the compound for conjugation reactions in phase II. The second phase of biotransformation involves the covalent linking of the activated toxic compound to specific hydrophilic molecules, which include glucose that binds hydroxyl, carboxyl and amino groups; malonate that binds amino and hydroxyl groups, and glutathione that binds to electrophilic sites on the activated compound (Coleman *et al.,* 1997). Conjugation of the activated toxic metabolite serves to, in some cases, increase the compound's water solubility and/or to deactivate the toxic compound. The result of phase II modifications is that the toxic metabolite or conjugate is significantly less phytotoxic, or not toxic at all. Phase II metabolites are thereafter compartmentalised within either the vacuole or apoplast (phase III). Chemical transformation or detoxification mechanisms in macrophytes are similar to those in animal cells, however, in macrophytes the transformed xenobiotic or conjugate is not excreted as in animal systems, but compartmentalised (Coleman *et al*., 1997).

An understanding of the metabolism, including possible detoxification of BMAA in macrophytes, not only aids in the understanding of its mechanisms of toxicity but also in understanding the risk of human exposure. This study is an investigation of the metabolism of stable-isotope labelled BMAA in the model aquatic macrophyte, *C. demersum*, via the analysis of label distribution patterns.

8.2 MATERIAL AND METHODS

8.2.1 BMAA exposure

Stable-isotope labelled BMAA (L-BMAA-4,4,4-d_3,$^{15}N_2$) was purchased from the Institute of Ethnomedicine (Jackson, Wyoming). *C. demersum* was purchased from Ultimate Aquatics (Port Elizabeth, South Africa) and to allow acclimatisation, was cultivated for four weeks prior to the commencement of the experiment in synthetic medium (Privasoli) (Pflugmacher *et al.,* 2006) at a constant temperature and illumination of 22 °C and 25 µmol E^{-2} s^{-1}, respectively.

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Replicate *C. demersum* sections (2 cm long), consisting of stem and leaves, were statically exposed in sterile water, each in an individual beaker, to 1 µM L-BMAA-4,4,4-d_3,$^{15}N_2$ (also referred to here as $^{5+}$BMAA) for 24 hours under illumination and temperature conditions as described above. Following 24 hours exposure, all specimens were individually removed from the exposure beakers and washed with sterile distilled deionised water followed by methanol. Each wash was repeated in triplicate, following which the washed specimens were placed in individual flasks containing only sterile distilled deionised water for depuration. Sections remained in BMAA-free medium (water) for 72 or 120 hours, after which they were harvested by snap freezing in liquid nitrogen and stored at -80 °C until further processing.

8.2.2 Extraction and analysis of amino acids

C. demersum sections were ground to a fine powder in liquid nitrogen and lyophilised overnight using a VirTis freeze dryer (−42.6 °C, 170 mTorr vacuum). For the extraction of free cellular amino acids, lyophilised plant matter was resuspended in 0.1 M trichloroacetic acid (TCA) by vortexing. Samples were thereafter sonicated in a water bath at 4 °C for 10 minutes and again vortexed. Sonication and vortexing was repeated once more, after which samples were incubated at room temperature for 30 minutes. Following incubation, samples were centrifuged (Eppendorf MiniSpin) at 10000 *x g* for 10 minutes at room temperature. The resultant protein pellet was washed in 0.1 M TCA by resuspension via vortexing. Following centrifugation as described above, the supernatant was pooled with that of the first extraction step. Protein-associated amino acids were extracted from the remaining protein pellet by acid hydrolyses with 6N HCl as described by Esterhuizen *et al.* (2011). Following hydrolysis, hydrolysates were filtered using Ultrafree-MC 0.22 mm centrifugal filtration units, the filtrate dried down using a Savant SpeedVac® and the dried residue resuspended in 20 mM HCl.

Free and protein-associated amino acid fractions were, following derivatisation with a propyl chloroformate derivative, separated using LC and analysed using single quadrupole mass spectrometry as described by Downing *et al*. (2012).

Detection and analysis of isotopically labelled amino acids were based on HPLC retention times and mass spectrometric detection of amino acid isotopologue parent ions as described by Downing *et al*. (2014). Analysis of label distribution from $^{5+}$BMAA to proteinogenic and non-proteinogenic amino acids was based on the relative abundance of amino acid isotopologues extracted from $^{5+}$BMAA-exposed cultures, normalised against control cultures. Due to the nature of this study, the aim being to investigate the metabolism of BMAA in *C. demersum* based on the redistribution of $^{5+}$BMAA labels, no absolute quantification of labelled or unlabelled amino acids was performed. Data represent the relative abundance of labelled amino acids based on LC/MS peak areas, unless otherwise stated.

8.3 RESULTS AND DISCUSSION

Labelled BMAA (BMAA-4,4,4-d_3,$^{15}N_2$) was taken up from the culture medium by *C. demersum,* with uptake data corresponding to previous studies (Esterhuizen *et al*., 2011) that showed concentration dependent uptake of exogenous BMAA by *C. demersum*, that followed Michaelis-Menton kinetics. Figure 8.1 shows the change in the free and protein-associated BMAA content of *C. demersum* over a 144 hour period during which the plants were exposed to 1 µM BMAA-4,4,4-d_3,$^{15}N_2$ for the initial 24 hours and thereafter transferred to BMAA-free sterile water for a 120 hour depuration period. During the depuration period there was a significant (p<0.05) reduction in intracellular BMAA, with nearly complete removal of free and protein-associated BMAA from the plants within 5 days. No BMAA was detected in the media during depuration. The decrease in cellular BMAA could therefore not be attributed to excretion, but was as a result of either complete catabolism or covalent modification of the amino acid. In other studies on BMAA uptake in macrophytes a marked decrease in cellular BMAA was also observed (Esterhuizen *et al*., 2011), even in plants continuously exposed to exogenous BMAA (Contardo-Jara *et al*., 2013). Detoxification mechanisms of xenobiotics in plants routinely involve covalent chemical modifications to the parent compound, yielding a conjugate or modified compound that is more hydrophilic and less toxic.

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These modified compounds are not excreted but compartmentalised within the cell (Coleman *et al.,* 1997). Therefore it is not particularly surprising that BMAA was not excreted during depuration, but as data suggest, rather sequestered inside the cell in a modified form. Furthermore, supporting the fact that BMAA was covalently modified in *C. demersum,* is the fact that there was very limited transfer of BMAA labels to other cellular amino acids. Figure 8.2 shows the changes in the abundance of amino acid isotopologues. The only significant increases in isotopologue abundance of any of the amino acids analysed were observed in arginine (Arg), which showed a 42 ± 24% increase in $^{1+}$Arg relative to $^{0+}$Arg following 24 hours of $^{5+}$BMAA exposure, and in asparagine (Asn) and aspartate (Asp) which showed a 35 ± 13% and 28 ± 17% increase, respectively, in the $^{2+}$isotopologue relative to the $^{1+}$isotopologue, following 120 hours of depuration.

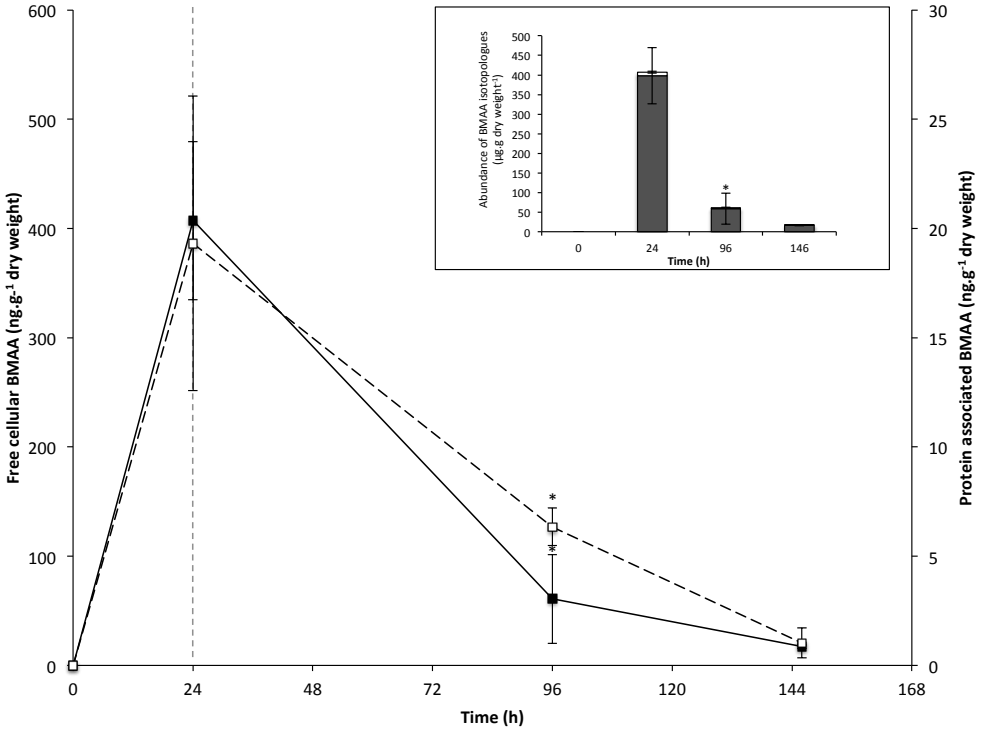

Figure 8.1: Free (solid line) and protein-associated (broken line) cellular BMAA in *C. demersum* over a total of 144 hours. Plant sections were exposed to 100 µM L-BMAA-4,4,4-d_3,$^{15}N_2$ for 24 hours, after which plants were washed and transferred (indicated by a dotted line) to BMAA-free water for depuration. (Insert) The abundance of BMAA isotopologues, where $^{5+}$BMAA is depicted by dark grey bars and $^{4+}$BMAA by open bars. Error bars depict standard deviations where n=3 and significant (p<0.05) differences from previous time points are indicated by an asterisk (*).

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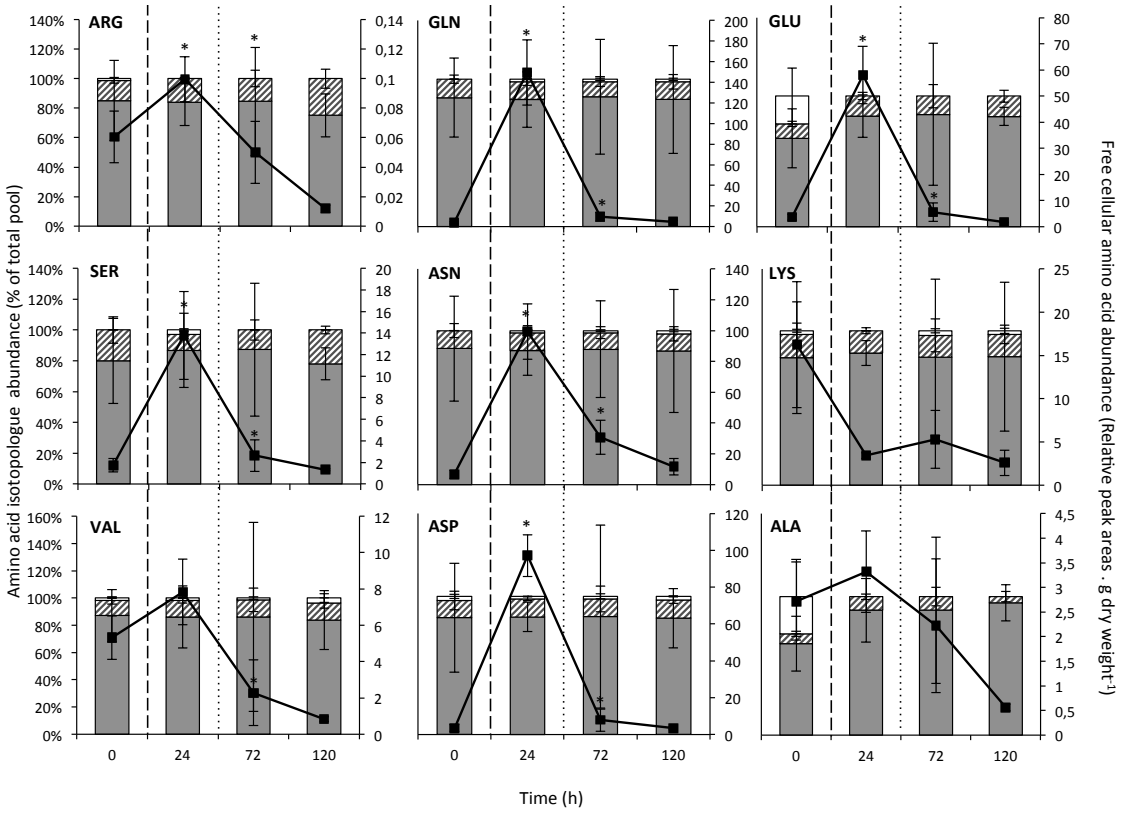

Figure 8.2: The change in amino acid isotopologue abundance in *C. demersum* exposed to 1 µM L-BMAA-4,4,4-d_3,$^{15}N_2$ for 24 h (dashed line indicates transfer of plants to BMAA medium), and during a 120 h depuration period (dotted line indicates transfer of plants to BMAA-free depuration medium). Bars show the abundance of amino acid isotopologues as a percentage of the total respective amino acid pool, where amino acid $^{0+}$isotopologues are indicated by grey bars, $^{1+}$isotopologues indicated by diagonally striped bars and $^{2+}$isotopologues are depicted by open bars. The total free cellular amino acid pool of respective amino acids is indicated by a solid line (secondary axis). All graphs depict LC/MS peak areas normalised against internal standard, methionine-d3. Error bars depict standard deviations where n=3 and significant (p<0.05) differences from previous time points are indicated by (**) for isotopologue ratios and by (*) for total free cellular amino acid pools.

Although these changes in the abundance of isotopes of these three amino acids are significant, the redistribution of $^{5+}$BMAA labels is minimal, and it does not in any way account for the substantial observed decrease of free cellular $^{5+}$BMAA. The redistribution of $^{5+}$BMAA labels most likely occurred during BMAA detoxification reactions, which may have resulted in the dissemination of chemical groups, free to be used in other anabolic processes such as amino acids biosynthesis. Therefore, based on the marked decrease of cellular BMAA over time in *C. demersum* and the negligible redistribution of $^{5+}$BMAA labels, data show that BMAA is not catabolised, nor does it

undergo any significant atomic exchanges such as de-methylation/methylation or de-amination/amination reactions, but that the neurotoxin is covalently modified and sequestered in the cells of *C. demersum*.

Following 24 hours exposure to L-BMAA-4,4,4-d_3,$^{15}N_2$ there was a significant increase in the free cellular pools of all amino acids analysed, except for lysine, where a significant decrease was observed (Figure 8.2). This increase in free cellular amino acids suggests either *de novo* amino acid synthesis or protein breakdown in response to $^{5+}$BMAA exposure. Analysis of total cellular protein before and after exposure did not show a significant decrease in the protein content of cells. That said, considering that the amount of free amino acids in plant cells is relatively low, a decrease in total cellular protein that is not significant relative to the entire cellular protein content, may still result in a significant increase in the amount of free amino acids. The increase in free amino acids cannot be attributed to the fact that BMAA served as a nutrient source, as data indicated that BMAA was not catabolised to any meaningful extent. As shown in Figure 8.2, all free amino acids analysed decreased significantly over the depuration period. This was expected as plants were depurated in nutrient free sterile water.

Various studies have confirmed the phytotoxicity of BMAA, with data showing increases in oxidative stress, enzyme inhibition (Contardo-Jara *et al*., 2013; Esterhuizen-Londt *et al.,* 2011) and plant development defects (Brenner *et al.,* 2000). Data from this study suggest that *C. demersum* responds to BMAA as a phytotoxin and not as a nutrient. Contardo-Jara *et al.* (2013) suggested that, due to its hydrophilic nature, BMAA would not be chemically transformed via cellular biotransformation systems that have been shown to biotransform the common cyanotoxin, microcystin, a system that involves the formation of a glutathione-microcystin conjugate catalyzed by glutathione *S*-transferase. However, chemical transformation of BMAA in plants via this or a similar biotransformation system should not be excluded based on the hydrophilic nature of BMAA. At physiological pH, due to the low pK_a value of the 2-amino group (Vega and Bell, 1967), BMAA is essentially uncharged and consequently very reactive (Nunn and Ponnusamy, 2009).

During the biotransformation of toxic compounds, given that a phytotoxin already contains a reactive group that can react with compounds during phase II, phase I of the biotransformation process can be bypassed (Coleman *et al.,* 1997). Therefore, considering the reactivity of BMAA under physiological conditions, it is very likely, as data suggest, that BMAA is covalently bound to some cellular molecule, resulting in the formation of a non-toxic BMAA-conjugate, which is sequestered within the plant, or perhaps, atypically for plant detoxification, modified and excreted in a modified form that was not detectable in the depuration medium.

8.4 CONCLUSION

Although the exact mechanism of BMAA detoxification in plants is still unknown, data from this study suggest that this cyanobacterial neurotoxin is covalently modified and compartmentalised within plants. This impacts significantly on the assessment of BMAA bioaccumulation in the environment as the inability to detect BMAA does not necessarily mean that the toxin is not present. Some studies have suggested that, during mammalian digestive processes, the toxic parent molecule or activated metabolites may be released from conjugates formed during biotransformation processes in plants (Sandermann, 1994; Sandermann 1992). Although, in this study, acid hydrolysis did not release significant amounts of BMAA, the possibility that other toxic metabolites can be released from BMAA-conjugates during mammalian digestion cannot be excluded. These data provide the first insights into the fate of the cyanobacterial neurotoxin BMAA in an aquatic plant, confirming that the molecule is not catabolised but covalently modified and stored within the cell.

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BMAA is covalently modified and sequestered in a non-BMAA form in the aquatic macrophyte *C. demersum*. Given that detoxification mechanisms in animals involve toxin conjugation and excretion rather than sequestration, the fate of BMAA in an aquatic animal model was investigated. An animal model was selected based on its relevance to the potential for human BMAA exposure.

CHAPTER 9: THE FATE OF THE CYANOBACTERIAL TOXIN β-*N*-METHYLAMINO-L-ALANINE IN FRESHWATER MUSSELS

ABSTRACT

The cyanobacterial neurotoxin, β-*N*-methylamino-L-alanine (BMAA) has been suggested as a causative agent for certain neurodegenerative diseases. This cyanotoxin bioaccumulates in an array of aquatic organisms, in which it occurs as both a free amino acid and in a protein-associated form. This study was intended to investigate the environmental fate of BMAA by examining the metabolism of isotopically labelled BMAA in four freshwater mussel species. All species showed substantial uptake of BMAA from the culture media. Data showed no significant evidence for BMAA catabolism in any of the animals but did suggest metabolism via the reversible covalent modification of BMAA in freshwater mussels, a process that appears to be variable in different species.

Keywords: β-*N*-methylamino-L-alanine, BMAA, metabolism, cyanobacteria, neurotoxin, mussels

9.1 INTRODUCTION

The bioaccumulation, biotransformation and depuration of cyanotoxins by aquatic invertebrates, including marine and freshwater mussels, have been studied extensively (Ferrão-Filho and Kozlowsky-Suzuki, 2011; Contardo-Jara *et al*., 2009; Fernandes *et al.*, 2009; Burmester *et al*., 2012). However, very few studies have focused on the cyanobacterial neurotoxin, β-*N*-methylamino-L-alanine (BMAA). This recently discovered cyanotoxin, first linked to free-living cyanobacteria in 2005 (Cox *et al*., 2005), elicits neurotoxicity via various mechanisms (reviewed in Chiu *et al.,* 2011), which include the induction of oxidative stress (Lui *et al.,* 2009) and misincorporation into proteins, resulting in protein truncation and aggregation (Rodgers and Dunlop*,* 2011). Various findings suggest a plausible link between the neurodegenerative disease Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex (ALS/PDC) and long term exposure to BMAA (Banack and Cox, 2003; Murch *et al.,* 2004; Caller *et al.,* 2009; Cox *et al.,* 2009).

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This putative link has prompted research into possible exposure routes of humans to this neurotoxin. Growing evidence confirms the environmental presence of BMAA in organisms other than cyanobacteria as a result of bioaccumulation and biomagnification, where the toxin is present both within the free cellular fraction and/or in association with proteins (Murch *et al.,* 2004). BMAA has been detected, although often in highly variable concentrations within various aquatic vertebrates and invertebrates, including mussels (Brand *et al.,* 2010; Jonasson *et al.,* 2010; Mondo *et al.,* 2012). To date, however, no information is available on the metabolic and consequent environmental fate of BMAA. The uptake by, and effect of cyanotoxins such as microcystins (MCs) and cylindrospermopsins (CYNs) on, freshwater mussels are well documented (Ferrão-Filho and Kozlowsky-Suzuki, 2011). However, inter-comparison of these studies is difficult due to variations in exposure regimes and form in which the toxin of concern was administered (whole toxin producing cyanobacterial cells, toxic cyanobacterial extracts, or pure toxin). Comparing the uptake by, and physiological response of, mussels to cyanotoxins such as MC and CYN to that of BMAA can also be difficult and perhaps irrelevant, because, unlike the relatively large and complex chemical structures of MC and CYN, BMAA is a simple neutral amino acid. Despite obvious contrasts in size and complexity, BMAA, like MC and CYN (Esterhuizen-Londt *et al.,* 2011), elicits toxicity through, amongst other mechanisms, oxidative stress via the induction of reactive oxygen species (ROS) formation. This mechanism of toxicity of BMAA is not specifically related to the amino acid nature of this toxin. However, other mechanisms of BMAA toxicity are more directly related to the fact that it is an amino acid. For example BMAA bicarbonate adducts, which spontaneously form in the presence of bicarbonate (Nunn and O'Brien, 1989), induce over-excitation of motor neurons by acting as glutamate analogs at excitatory ionotropic glutamate receptors (Weiss and Choi, 1988; Weiss *et al*., 1989). An additional proposed mode of toxicity involves the erroneous incorporation of BMAA into *de novo* synthesised proteins, causing protein truncation and misfolding (Rodgers and Dunlop, 2011). Because some of the mechanisms of BMAA toxicity function due to the fact that it is treated as an amino acid by cells, any investigation of the fate of BMAA in mussels should be based not only on typical xenobiotic/toxin metabolism but also on typical amino acid metabolism.

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Amino acid uptake in mussels is catalyzed by various amino acid transport enzymes, which vary in amino acid affinity and specificity (Wright, 1985; Baines *et al.,* 2005). Baines *et al.* (2005) showed that uptake of exogenous amino acids in *Dreissena polymorpha* varied significantly depending on the nature of the molecule. Less than 5% of alanine remained in the culture media after only 4 h exposure compared to more than 85% glutamic acid remaining in the medium after 8 h exposure. Furthermore, according to Wright and Pajor (1989) and Preston (1993), marine mussels are capable of accumulating dissolved organic matter (DOM), including amino acids, against a concentration gradient, utilizing Na^+ co-transport systems. However the paucity of reports on amino acid or DOM uptake by freshwater mussels may be attributed to the lack of such co-transport systems (Baines *et al.,* 2005). Nevertheless, recent studies have shown that *D. polymorpha*, is capable of efficient uptake of amino acids from its environment (Baines *et al.,* 2005). At the time of the study this ability of *D. polymorpha* was considered unique among all freshwater mussels in which uptake of dissolved organic matter had been investigated. This trait has also been suggested as a reason for the species' environmental persistence in conditions often unfavorable for the sustainability of other native species (Baines *et al.,* 2005).

As growing evidence suggests a link between exposure to environmental BMAA and neurodegeneration, it has become increasingly important to establish the risk of BMAA exposure. Thus, in this study we aimed to investigate the environmental fate of BMAA by evaluating its uptake, metabolism and possible depuration in four freshwater mussel species.

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9.2 MATERIAL AND METHODS

9.2.1 Mussels and mussel husbandry

Four freshwater mussel species, *Anadonta cygnea* (70–90 mm), *Unio tumidus* (70-90 mm), *Dreissena polymorpha* (25–30 mm) and *Corbicula javanicus* (23–25 mm) were used in this investigation. *A. cygnea* and *U. tumidus* were obtained from Förde Fisch (Germany), *D. polymorpha* was collected from Großer Küstrinsee, a semipristine area 100 km north of Berlin, Germany, and *C. javanicus* were purchased from Zoo Zajak (Germany). All mussels were acclimatised over a 2 week period prior the experiments to the laboratory conditions. Whereas *A. cygnea* and *U. tumidus* were kept in 50 L aerated glass aquaria with 20 individuals per tank, *D. polymorpha* and *C. javanicus* were kept in 10 L aerated glass aquaria with 50 individuals per tank. *A cygnea, U. tumidus* and *C*. *javanicus* received a 10 cm sediment (particle size 0.2–6 mm) layer to allow burrowing during acclimatisation. All mussels were held at 22 ± 1 °C under a constant day:night cycle of 14:10 h and fed daily with freeze-dried *Spirulina* sp. powder. The mussel culture medium, used in both routine maintenance and during $^{5+}$BMAA exposure experiments, consisted of Milli-Q-grade water containing 0.106 g L^{-1} $NaHCO_3$ (Carl Roth, Z99%), 0.2 g L^{-1} $CaCl_2$ (Carl Roth, Z99%) and 0.1 g L^{-1} sea salt (Aqua medic).

9.2.2 BMAA exposure setup

Uptake, metabolism and depuration of exogenous BMAA were investigated by exposing mussels to ~100 µg L^{-1} isotopically labelled BMAA; L-BMAA-4,4,4-d3,$^{15}N_2$ (donated by the Institute of Ethnomedicine, at a purity of 99%), also referred to here as $^{5+}$BMAA, for 24 or 48 h, followed by a 24 h depuration period. All mussels were cultured in individual aquaria throughout the duration of the experiment. Different culture media volumes for BMAA exposure purposes were selected to allow equivalence in tissue-volume:exposure-medium-volume ratios. Thus, whereas the larger mussels *U. tumidus* and *A. cygnea* were exposed to BMAA in a total volume of 4 L, the smaller species *C*. *javanicus* and *D. polymorpha* were exposed to $^{5+}$BMAA in a culture volume of 600 mL.

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For each species the experimental set-up included 24 replicates (each replicate cultured in individual aquaria). Eight mussels were cultured in the presence and four mussels in the absence of exogenous $^{5+}$BMAA (control mussels) for 24 h. Following the 24 h, four replicates grown in the presence of $^{5+}$BMAA were washed with BMAA-free culture media and transferred to identical aquaria containing $^{5+}$BMAA-free culture media for further 24 h depuration. The remaining replicates and control mussels were dissected on ice, the soft tissue snap-frozen in liquid nitrogen and stored at -80 °C until further processing. A further eight mussels were cultured in the presence and four mussels in the absence of exogenous $^{5+}$BMAA for 48 h. Following the 48 h, four replicates grown in the presence of $^{5+}$BMAA were transferred to BMAA-free media for further 24 h depuration as described above. The remaining mussels were sampled as previously described.

9.2.3 Sample preparation and analysis

Free and protein-associated amino acid extraction

Free and protein-associated amino acids were extracted from wet mussel tissue by homogenising mussel soft tissue in 0.1 N trichloroacetic acid (TCA) (Sigma-Aldrich, for HPLC, ≥99%). Homogenates were incubated with constant agitation for 30 min at room temperature, after which protein pellets were collected via centrifugation at 4000 *x g* for 10 min. Protein pellets were washed with 0.1 N TCA and collected again via centrifugation as described above. Supernatants, containing the free amino acid fraction, from TCA homogenate and TCA wash were pooled and stored at -80 °C until further processing.

Protein pellets were subsequently hydrolyzed in 6 N HCl (Merck, Pro Analysis, fuming 37%) at 110 °C for 18 h. The protein hydrolysate was filtered using Ultrafree-MC 0.22 mm centrifugal filtration units. The filtrate was then dried down using a Savant Speed Vac Plus and the residue re-suspended in 20 mM HCl. All samples were stored at -80 °C until further processing.

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HPLC/MSMS sample analysis

Following pre-column derivatisation using a propyl chloroformate derivative as described by Downing *et al.* (2012), free and protein-associated amino acid extracts were analysed by liquid chromatography/mass spectrometry (LC/MS) using an Alliance 2695 UHPLC combined with a Micromass Quattro micro™, Waters. Derivatized samples (injection volume of 1 µL) were separated using a Phenomenex EZ:faast AAA-MS 250 x 2.00 mm amino acid analysis column (Phenomenex, Torrance, USA) by linear gradient elution (0.00 min 68% B, 13.00 min 83% B, 13.01 min 68% B, 17.00 min 68% B) with a mobile phase composition of (A) 10 mM ammonium formate (Carl Roth, ≥98% Pro Analysis ACS) in water (VWR, HPLC Super Gradient Grade) and (B) 10 mM ammonium formate in methanol (VWR, HPLC-MS Grade) at a flow rate of 0.25 mL min^{-1} and a column temperature of 35 °C.

$^{5+}$BMAA was quantified using electron spray ionization (ESI) positive ion mode single quadrupole mass spectrometry with a source temperature of 150 °C, a scan range of 200–450 *m/z*, a capillary voltage of 5 kV and a cone voltage of 20 V. Nitrogen was used as cone gas at a flow rate of 15 L h^{-1} and as desolvation gas at 250 L h^{-1} with a desolvation temperature of 250 °C. The presence of $^{5+}$BMAA in samples was confirmed using ESI positive ion mode triple quadrupole mass spectrometry (multiple reaction monitoring) with a source temperature of 120 °C, and capillary voltage of 5 kV and a cone voltage of 20 V. Nitrogen was used as cone gas at a flow rate of 30 L h^{-1} and as desolvation gas at 40 L h^{-1} with a desolvation temperature of 200 °C. Argon was used as collision gas at 13 V. The parent ion 338 *m/z* (derivitised $^{5+}$BMAA) was selected in Q1 and the subsequent product ions 278, 250 and 235 *m/z*, produced during collision-induced ionization were selected by Q3. Data analysis was conducted with the software package MassLynx. Calibration was linear (R^2=0.999) between 5 and 300 ng mL^{-1}. Calibration standards were prepared in 20 mM HCl. LOD was 1 ng mL^{-1} derivatized $^{5+}$BMAA.

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Analysis of the metabolism of isotopically labelled BMAA

Metabolism of $^{5+}$BMAA was investigated based on the redistribution of constituent stable isotopes (15N or 2H) to other amino acids (Glu, Gln, Asp, Asn, Cys, Trp, Ala, Orn, Gly, Ser, Lys, Val, Leu), both within the free and protein-associated amino acid fractions. Analysis of the incorporation of stable isotopes into *de novo* synthesized cellular amino acids was based on HPLC retention times and mass spectrometric detection (Waters Micromass Quattro micro™ operating in Q1 mode, with no collision-induced dissociation of parent ions) of parent ions for all possible isotope label additions for each amino acid analysed. Analysis of the incorporation of $^{5+}$BMAA-originating isotopes into amino acids was based on the abundance of isotope-containing amino acids relative to non-isotopically labelled amino acids extracted from mussels that had been exposed to isotopically labelled BMAA, normalised against control, non-exposed mussels. Due to the nature of this study, the aim being to investigate the metabolism of BMAA in freshwater mussels, based on the redistribution of $^{5+}$BMAA-originating isotopes, no quantification of amino acids (other than $^{5+}$BMAA) was performed. In order to guage the extent of $^{5+}$BMAA-originating isotope redistribution, LC/MS data were processed to reflect changes in the ratios of ion $m/z + x$ to ion m/z (where x = 1,2,3,4 or 5), relative to this ratio in control samples. For example, glutamate could obtain a maximum of four isotopes from BMAA. LC/MS analysis would detected the presence of native glutamate and, if present, the presence of other glutamate isotopes, $^{1+}$Glu, $^{2+}$Glu, etc. An increase in the ratio $^{1+}$Glu:Glu relative to control samples not exposed to isotopically labelled BMAA would indicate a redistribution of molecular isotopes from $^{5+}$BMAA to glutamate.

9.2.4 Statistical analysis

Statistical significance of variance was determined by means of Student's *t* test (two-sample assuming unequal variances) using Statistica 9.0 (StatSoft Inc., USA).

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9.3 RESULTS AND DISCUSSION

9.3.1 Uptake of exogenous BMAA

Quantification of $^{5+}$BMAA uptake was undertaken to confirm the internalisation of exogenous $^{5+}$BMAA and its intracellular presence. Analysis of cellular $^{5+}$BMAA also provided information regarding the cellular fate of $^{5+}$BMAA following internalisation. Specifically, does the neurotoxin remain within the free amino acid fraction, or does $^{5+}$BMAA polymerise and/or become associated with proteins?

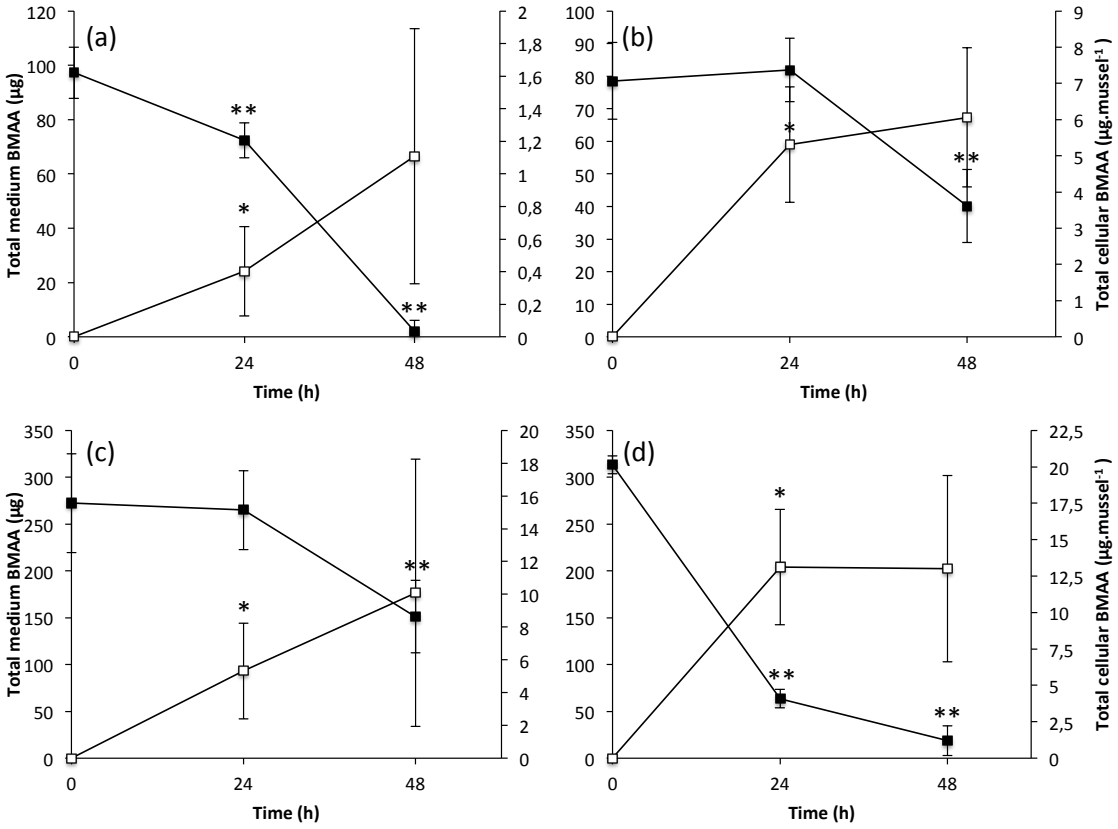

Figure 9.1: The uptake of exogenous isotopically labelled BMAA by (a) *D. polymorpha* (b) *C. javanicus*, (c) *A. cygnea* and (d) *U. tumidus* over 48 h, expressed as the change in total available exogenous medium $^{5+}$BMAA (solid squares) and the change in total cellular $^{5+}$BMAA in total mussel soft tissue (open squares). Error bars denote standard deviations, where n=4. Significance, p<0.05, is indicated by (*) for cellular $^{5+}$BMAA and by (**) for medium $^{5+}$BMAA.

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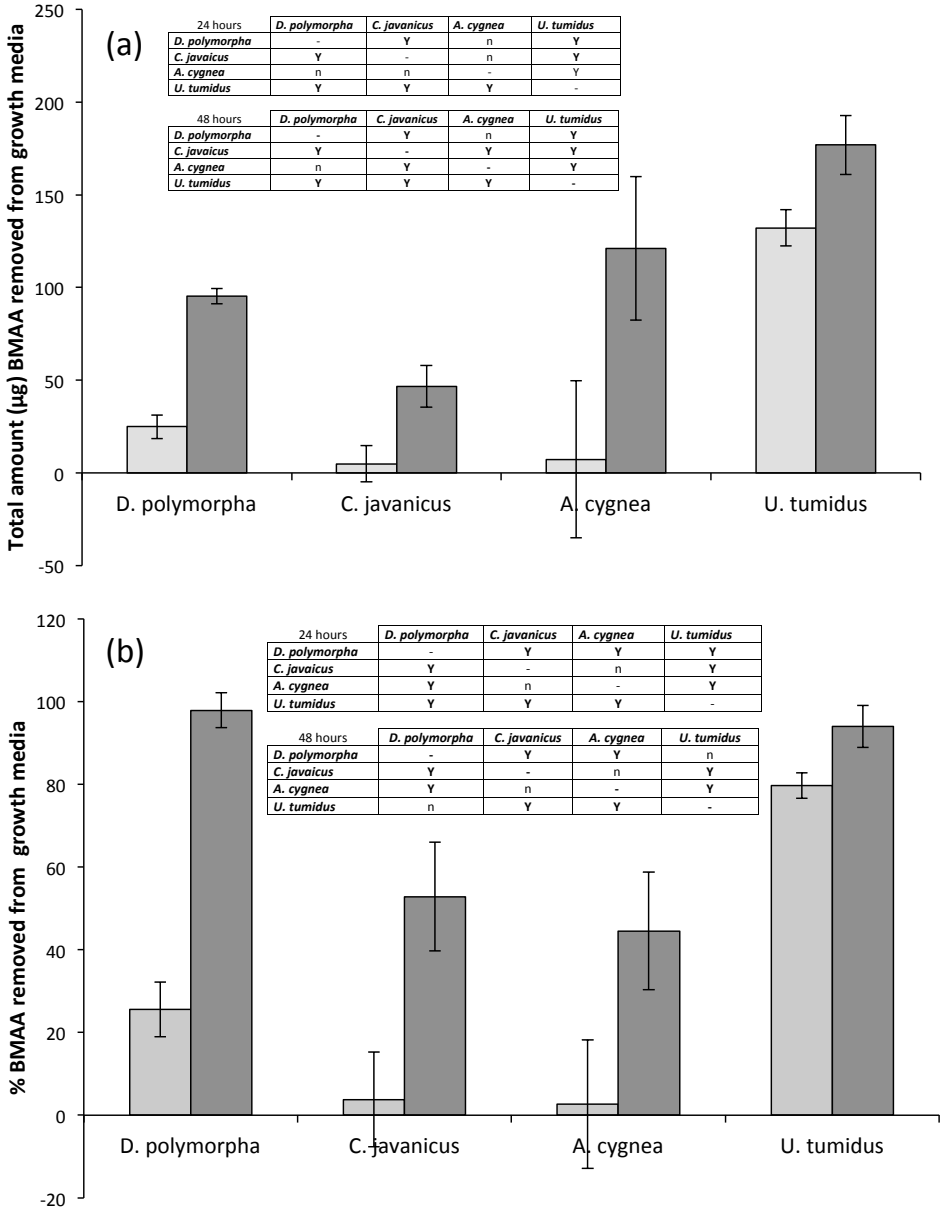
Isotopically labelled BMAA was taken up by all four freshwater mussel species tested; *D. polymorpha, C. javanicus, A. cygnea* and *U. tumidus* (Figure 9.1). In all species except *U. tumidus* the rate of $^{5+}$BMAA uptake was substantially lower within the first 24 h of exposure compared to uptake rates during the 24 h that followed.



24 hours

	D. polymorpha	C. javanicus	A. cygnea	U. tumidus
D. polymorpha	-	Y	n	Y
C. javaicus	Y	-	n	Y
A. cygnea	n	n	-	Y
U. tumidus	Y	Y	Y	-

48 hours

	D. polymorpha	C. javanicus	A. cygnea	U. tumidus
D. polymorpha	-	Y	n	Y
C. javaicus	Y	-	Y	Y
A. cygnea	n	Y	-	Y
U. tumidus	Y	Y	Y	-

24 hours

	D. polymorpha	C. javanicus	A. cygnea	U. tumidus
D. polymorpha	-	Y	Y	Y
C. javaicus	Y	-	n	Y
A. cygnea	Y	n	-	Y
U. tumidus	Y	Y	Y	-

48 hours

	D. polymorpha	C. javanicus	A. cygnea	U. tumidus
D. polymorpha	-	Y	Y	n
C. javaicus	Y	-	n	Y
A. cygnea	Y	n	-	Y
U. tumidus	n	Y	Y	-

Figure 9.2: Differences in BMAA uptake efficiency in; *D. polymorpha*, *C. javanicus*, *A. cygnea* and *U. tumidus*, expressed as (a) the total amount (µg) of $^{5+}$BMAA removed from the growth media and (b) the percentage $^{5+}$BMAA removed from the growth media, after 24 (light grey bars) or 48 h (dark grey bars) exposure to isotopically labelled BMAA. Error bars denote standard deviations, where n=4. In all species uptake of $^{5+}$BMAA at 48 h was significantly (p<0.05) greater than that at 24 h. Significant (p<0.05) inter-species differences at 24 and 48 h are indicated by "Y" in the table inserts, "n" indicates no significant difference.

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Visual observation confirmed that all individual mussel replicates were actively filtering within an hour of being transferred into aquaria containing isotopically labelled BMAA and were observed to be actively filtering at each sampling time.

Although the uptake of $^{5+}$BMAA within 48 h was substantial in all species the total amount of $^{5+}$BMAA removed from the culture medium varied significantly (p<0.05) between mussel species (Figure 9.2a). *D. polymorpha* and *U. tumidus* removed nearly all of the exogenous $^{5+}$BMAA available in the respective aquaria (Figure 9.2b), 98 ± 4% and 94 ± 5% respectively, within 48 h, compared to 53 ± 13% and 45 ± 14% of the total available $^{5+}$BMAA removed by *C. javanicus* and *A. cygnea*, respectively, within the same time frame. *A. cygnea* and *U. tumidus* have considerably greater filtration capacities compared to that of *D. polymorpha* and *C. javanicus*. Kryger and Riisgård (1988) reported the filtration capacity of *U. tumidus* to be 2.5–3.4 times higher per hour than that of *D*. *polymorpha.* Based on filtration capacity, it would therefore be reasonable to expect that *A. cygnea* and *U. tumidus* would remove more $^{5+}$BMAA from the medium relative to that removed by *D. polymorpha* and *C. javanicus*. This held true for *C. javanicus*, which removed significantly (p<0.05) less $^{5+}$BMAA from the medium over a total period of 48 h compared to that of *A. cygnea* and *U. tumidus* (Figure 9.2). Understandably, due to the greater volume of the aquaria housing the larger mussel species, the total amount of $^{5+}$BMAA available for uptake to these species was ~3 times higher than that available for uptake in smaller aquaria housing *D. polymorpha* and *C. javanicus*. Although the amount of $^{5+}$BMAA removed by *D. polymorpha* was significantly (p<0.05) less than that removed by *U. tumidus* at 24 and 48 h, it was comparable to the amount removed by *A. cygnea* (Figure 9.2). Given the substantial uptake of BMAA by *D. polymorpha*, it is conceivable that this species would have continued to remove $^{5+}$BMAA from the medium given that there was a greater amount available for uptake. The filtration capacity of *A. cygnea* may have been reduced as the filtration rate of burrowing mussels, such as *A. cygnea*, can be limited by the absence of sediment (Kryger and Riisgård, 1988). In addition, assuming that BMAA is taken up via constituent amino acid transport proteins (Baines *et al.,* 2005) differences in the affinity for BMAA of amino acid transport enzymes in different mussel species may account, in part, for the observed differences in BMAA uptake.

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Furthermore, mussels in which $^{5+}$BMAA uptake was most substantial (*U. tumidus* and, relative to its size, *D. polymorpha*) may possess a mechanism enabling them to better modulate the accumulation of this cyanotoxin.

Following 48 h exposure the concentration of $^{5+}$BMAA in all mussels was markedly lower than published data on BMAA concentrations recorded in environmental marine mussels (Jonasson *et al*., 2010 and Brand *et al*., 2010). The highest $^{5+}$BMAA concentrations (μg g^{-1} wet weight) recorded in mussels exposed to $^{5+}$BMAA were ~50–80 times lower than BMAA concentrations (μg g^{-1} dry weight) recorded in *Mytilus edulis* from the Baltic sea by Jonasson *et al.* (2010) and ~13 x 10^4 to 16 x 10^4 times lower than BMAA concentrations (μg g^{-1} dry weight) recorded in *Utterbackia imbecillis* from the Caloosahatchee River by Brand *et al.* (2010). Due to the inefficiency of energy generating co-transport systems in freshwater mussels, amino acid accumulation in these animals can be substantially lower than that of marine mussels (Baines *et al.,* 2005). This, amongst other factors such as the duration of BMAA exposure and environmental factors such as temperature, nutritional state, sex and variation in toxin analysis methods may account for such differences.

9.3.2 BMAA depuration and metabolism

Data in Figures 9.3 and 9.4 highlight the discrepancies between the total amounts of $^{5+}$BMAA removed from the culture media and the total amounts of $^{5+}$BMAA detected in the organisms. Such discrepancies, expressed as “unaccounted for BMAA”, could not be explained by $^{5+}$BMAA loss from the culture medium due to other non-mussel related biological activity, such as natural or microbial degradation. Loss of BMAA from control aquaria (containing no mussels) was not significant over four days. Although not measured in this study, Baines *et al.* (2005) showed that the amount of dissolved organic matter (including amino acids) taken up by bacteria associated with freshwater mussels was always <1% of the total amount originally dissolved within the culture media. Based on this, the fraction of unaccounted for $^{5+}$BMAA attributed to microbial uptake and/metabolism can be considered negligible. The discrepancy between the amounts of

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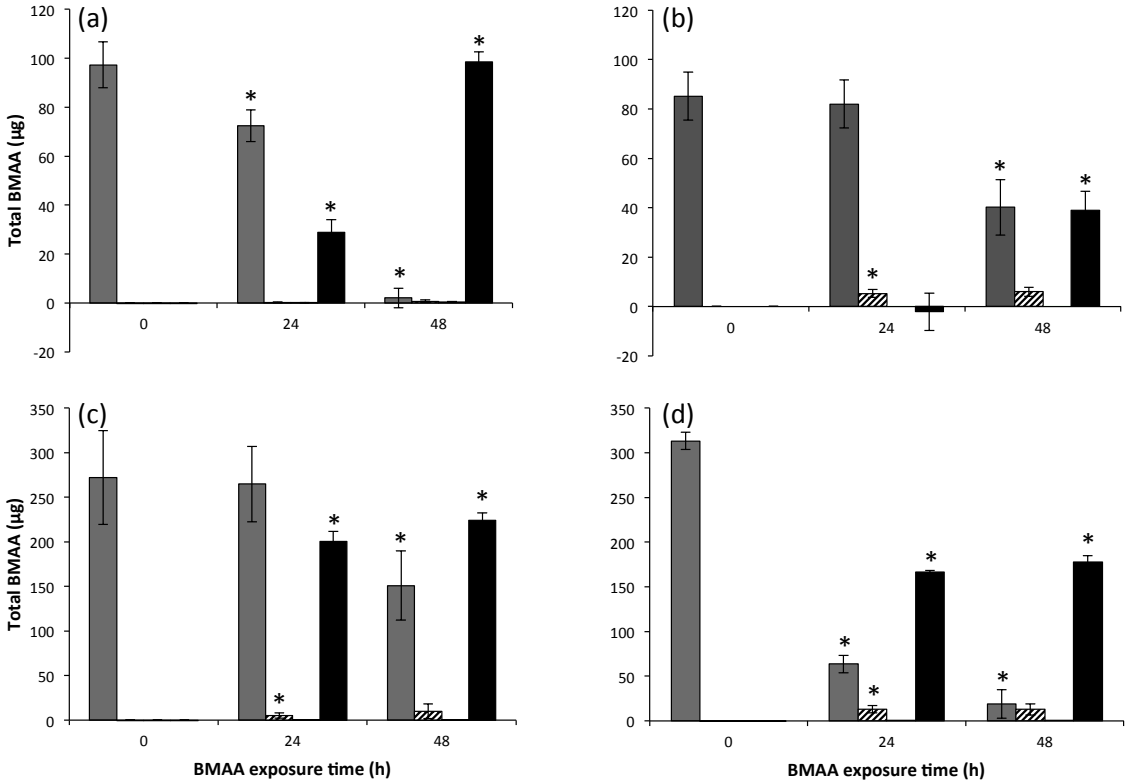

Figure 9.3: The distribution of exogenous isotopically labelled BMAA following exposure to (a) *D. polymorpha* (b) *C. javanicus*, (c) *A. cygnea* and (d) *U. tumidus* over 48 h, expressed as the change in total $^{5+}$BMAA in the growth medium (grey bars), in the free cellular amino acid fraction (diagonally striped), and in the protein-associated amino acid fraction (open bars). Unaccounted for $^{5+}$BMAA as calculated from the total $^{5+}$BMAA originally in the growth medium prior to addition of mussels is depicted by solid black bars. Error bars denote standard deviations, where n=4. Significant differences, p<0.05, from previous time points are indicated by (*).

$^{5+}$BMAA lost from the media and $^{5+}$BMAA within the exposed organism was most prominent in *D. polymorpha*, in which only ~1.4% of the total amount of $^{5+}$BMAA removed from the culture media was accounted for in the total cellular $^{5+}$BMAA fraction. This discrepancy is clearly illustrated in Figure 9.4, which also shows the differences in the cellular distribution and/or fate of internalised $^{5+}$BMAA in different mussel species.

The large discrepancy between $^{5+}$BMAA loss from the culture medium and the actual amount of $^{5+}$BMAA within mussel tissue, suggests that following uptake of $^{5+}$BMAA the neurotoxin either associates with proteins, is catabolised, covalently modified or directly excreted. Feces production by *U. tumidus* and *C. javanicus* was inconsistent amongst

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replicates and absent in some cases. Feces were collected from mussel species (*A. cygnea* and *D. polymorpha*) in which sufficient amounts for extraction of amino acids were produced during the sampling period. In aquaria housing *A. cygnea*, there was no significant difference between the $^{5+}$BMAA concentrations of the exposure medium and that of feces excreted within either 24 or 48 h exposure to exogenous $^{5+}$BMAA. However, following 24 h depuration, the $^{5+}$BMAA content of the feces excreted by *A. cygnea* was markedly higher compared to the medium in aquaria housing mussels exposed to exogenous $^{5+}$BMAA for 24 and 48 h. Furthermore, the amount of $^{5+}$BMAA in the feces excreted during 24 h depuration by mussels exposed to $^{5+}$BMAA for either 24 or 48 h was markedly higher than the amount of cellular $^{5+}$BMAA within the mussels at the commencement of depuration.

Although these differences were not statistically significant, it does suggest that BMAA was present within the animals in a modified form, and that this modification was reversed prior to excretion of $^{5+}$BMAA in the feces. In *D. polymorpha*, $^{5+}$BMAA concentrations in the medium and feces were only significantly (p<0.05) different following 48 h exposure to BMAA. At 48 h, 98% of the total amount of medium $^{5+}$BMAA had been removed from the culture medium. On average a total of 10 ± 4 mg of $^{5+}$BMAA was excreted in the feces by *D. polymorpha* following 48 h $^{5+}$BMAA exposure. Although a fairly substantial amount of $^{5+}$BMAA was excreted in the feces of *D. polymorpha*, the amount of $^{5+}$BMAA in the feces does not account for the total of $^{5+}$BMAA unaccounted for. Amorim and Vasconcelos (1999) observed a substantial excretion of MC via the feces in *Mytilus galloprovincialis*, though it must be noted that MC was administered in the form of whole cyanobacterial cells, which can remain undigested resulting in the excretion of whole toxin-containing cells. Nevertheless, in the current study, excretion of $^{5+}$BMAA through the feces may account for a portion of $^{5+}$BMAA unaccounted for. With longer depuration it is conceivable that the remainder of the unaccounted for $^{5+}$BMAA may convert back into $^{5+}$BMAA and excreted.

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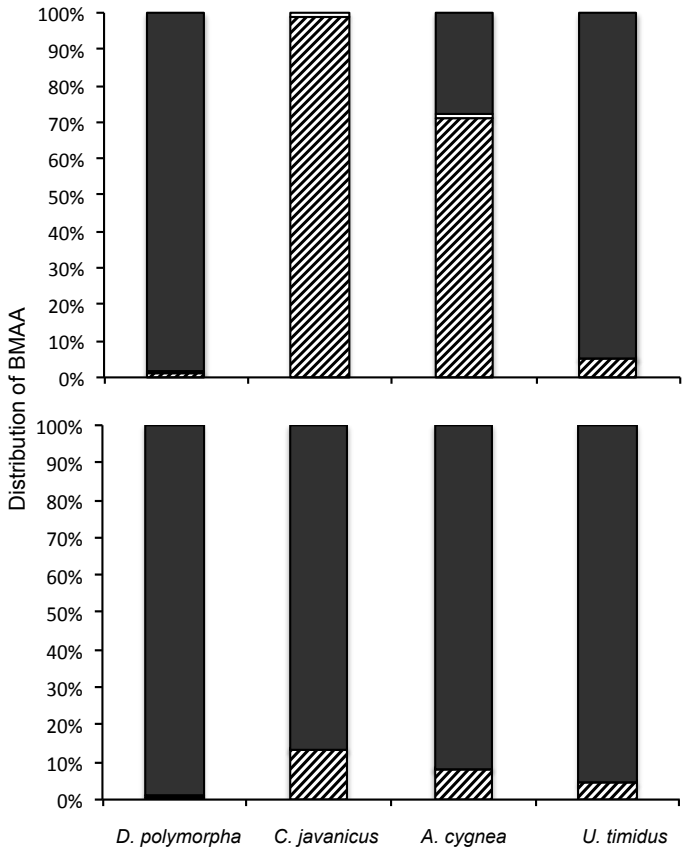

Figure 9.4: The fate of exogenous $^{5+}$BMAA following uptake by freshwater mussels *D. polymorpha*, *C. javanicus*, *A. cygnea* and *U. tumidus* at 24 h (upper pane) and 48 h (lower pane) after exposure to isotopically labelled BMAA. The graph depicts free cellular $^{5+}$BMAA (diagonally striped), protein-associated $^{5+}$BMAA (open bars) and $^{5+}$BMAA not accounted for (dark grey bars) as a percentage of the total amount of $^{5+}$BMAA removed from the media in 24 or 48 h.

9.3.3 Redistribution of BMAA-originating molecular isotopes

LC/MS analysis of free and protein-associated amino acid extracts revealed the presence of amino acid isotopologues in the expected amounts for the natural abundance of molecular isotopes as previously observed by Downing *et al.* (2011). There was no significant increase in the abundance of amino acid isotopologues in free or protein-associated amino acid fractions isolated from mussels exposed to isotopically labelled BMAA, relative to that of control mussels not exposed to isotopically labelled BMAA. Therefore no redistribution of $^{5+}$BMAA-originating isotopes to *de novo* synthesised amino acids took place, which indicates that $^{5+}$BMAA was not metabolised via transamination reactions or catabolised via deamination or demethylation reactions in any of the mussel species tested within a period of 72 h. A study by Baines *et al*. (2005)

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showed that in *D. polymorpha* ~60% of ^{14}C acquired through the uptake of ^{14}C–alanine from solution was present in the dissolved inorganic fraction within an 8 h period, while 20% remained within the soft tissue of the mussel. Based on this, although $^{5+}$BMAA catabolism was not observed in the current study, the timeframe of exposure was adequate to allow amino acid catabolism.

Apart from the usual transamination, deamination or demethylation reactions, it is also possible that BMAA is metabolised via non-amino acid pathways. However, cellular metabolism is a stringently controlled process during which essential macromolecules are continuously recycled. Therefore, irrespective of the pathway of BMAA metabolism, if this cyanotoxin were catabolised in these organisms, the prevalence of amino acid isotopes would ultimately increase as the $^{5+}$BMAA-originating nitrogen or hydrogen isotopes were cycled through the cell. This was not observed over a 72 h period.

9.3.4 BMAA depuration

Figure 9.5 shows the cellular distribution of $^{5+}$BMAA within mussels during a 24 h depuration period following exposure to isotopically labelled BMAA for 24 h (Figure 9.5 (1), left pane) and 48 h (Figure 9.5 (2), right pane). A significant (p<0.05) and substantial (74 ± 6 µg and 50 ± 13 µg, respectively) increase in isotopically labelled BMAA was observed within 24 h in the media of aquaria containing *C. javanicus* (b) and *U. tumidus* (d) that had been exposed to isotopically labelled BMAA for 24 h. A significant (p<0.05) but less substantial (33 ± 13 µg and 15 ± 10 µg, respectively) increase in media $^{5+}$BMAA was observed in aquaria containing *C. javanicus* and *U. tumidus* that had been exposed to isotopically labelled BMAA for 48 h. No $^{5+}$BMAA was detected in the media during the same depuration period in aquaria containing *D. polymorpha* and *A. cygnea*. Studies on the depuration of MC in the marine mussel *M. galloprovincialis*, also showed release of the toxin back into the depuration media within 2 days (Amorim and Vasconcelos, 1999), which was attributed to MC excreted in the urine.

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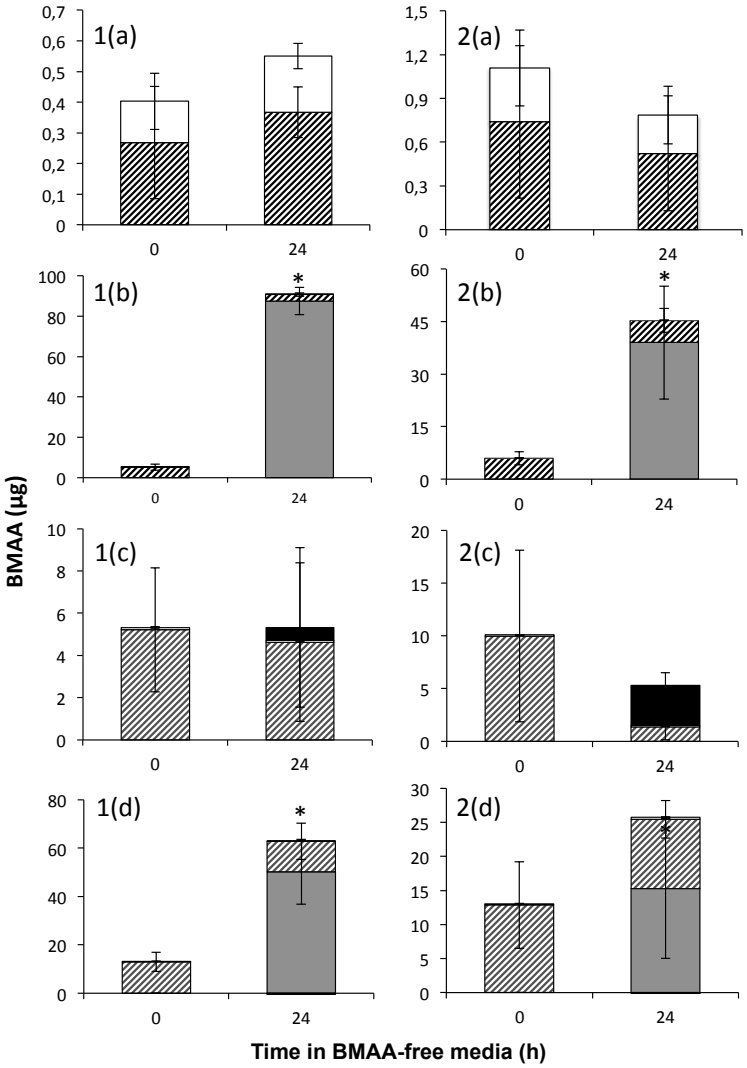

Figure 9.5: The fate of exogenous $^{5+}$BMAA following uptake by freshwater mussels *D. polymorpha* (a), *C. javanicus* (b), *A. cygnea* (c) and *U. tumidus* (d). Data show the distribution of internalised $^{5+}$BMAA during a 24 h depuration period, following 24 h (1) or 48 h (2) exposure to $^{5+}$BMAA. The figure shows the total amount of isotopically labelled BMAA placed into the depuration media (in the form of intracellular $^{5+}$BMAA) depicted as a stacked bar (0 h) and its distribution following 24 h, where free cellular (diagonally striped bars), protein-associated (open bars), media $^{5+}$BMAA (light grey bars) and unaccounted for $^{5+}$BMAA (black bars). Error bars denote standard deviations where n=4, and significant (p<0.05) differences from previous time points are indicated by (*).

$^{5+}$BMAA release into the medium via urine is likely and could account for some of the BMAA detected in the depuration medium. However, remarkably, the amount of $^{5+}$BMAA released into the media by these mussels was nearly 5–15 times higher than the amount of $^{5+}$BMAA present in the mussel soft tissue at the beginning of the depuration period. This suggests that $^{5+}$BMAA was released from the mussel soft tissue where it was present in a form undetectable by routine methods.

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Such fluctuations or discrepancies in the amount of detectable toxin have also previously been observed during depuration studies on MC in *M. galloprovincialis* (Amorim and Vasconcelos, 1999) and on CYN in *A. cygnea* (Saker *et al*., 2004), during which the amounts of cellular toxin increased during the depuration period. Amorim and Vasconcelos (1999) attributed such fluctuations to the fact that MC covalently associates with protein phosphatase enzymes becoming undetectable by the methods of detection used in their analysis. BMAA does associate with proteins (Murch *et al.,* 2004) and may be erroneously incorporated into proteins (Rodgers and Dunlop, 2011). However, if protein–$^{5+}$BMAA associations did occur within mussel soft tissue, as suggested for MC, such sequestered $^{5+}$BMAA would still be detectable following acid hydrolysis of cellular protein pellets. This was not observed. Therefore, the data suggest that $^{5+}$BMAA was reversibly covalently modified.

Further evidence that supports the covalent modification of exogenous $^{5+}$BMAA is the large discrepancy, not accounted for by catabolism, or natural degradation of the amino acid, that exists between $^{5+}$BMAA removed from the media and that detected inside the organisms (Figures 9.3 and 9.4).

9.3.5 BMAA metabolism and implications for its environmental prevalence

Amino acid uptake in mussels follows Michaelis–Menton kinetics (Barker Jørgensen, 1983), consequently uptake of an amino acid is limited by its cellular concentration. The ability of mussels to reversibly modify a molecule, in this case the amino acid BMAA, means that the organism can essentially reduce the intercellular concentration of the molecule. As previously mentioned, the uptake of dissolved organic matter, here more specifically amino acids, against a concentration gradient is challenging without an energy yielding co-transport system. Assuming that BMAA is internalised by amino acid transporter proteins, the covalent modification of BMAA reduces the intracellular BMAA concentration, enabling its continual uptake. Such metabolism of BMAA may account for the ability of *D. polymorpha* to remove $^{5+}$BMAA from the media to an extent similar to that of the much larger mussel as *A. cygnea*, which has a much greater filtration capacity

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compared to that of *D. polymorpha*. Additionally, the efficiency of BMAA metabolism may vary between different species.

The metabolism of cyanotoxins and other xenobiotics by mussels is not uncommon. In a study by Contardo-Jara *et al.* (2011) a major physiological response was observed in *D. polymorpha* exposed to the pharmaceutical Bezafibrate (BEZ), however no BEZ was detected in the mussel tissue. The authors suggested that this discrepancy might be attributed to possible BEZ metabolism by *D. polymorpha*. Metabolism of the pharmaceutical, 17-β-estradiol, was observed by Le Curieux-Belfond *et al.* (2005) in oysters. Burmester *et al.* (2012) compared the capacity of *D. polymorpha*, and *U. tumidus* to metabolise the cyanotoxin MC via biotransformation, hence enhancing the water solubility by appending cellular macromolecules to the compound. However, although both organisms were capable of MC biotransformation, the authors could not conclude whether or not *D. polymorpha* is better adapted to deal with cyanotoxin exposure compared to *U. tumidus*. Since BMAA is hydrophilic, biotransformation of this amino acid is not expected by the primary biotransformation enzymes cytochrome P-450 and glutathione *S*-transferase. Data from the current study showed that both *D. polymorpha* and *U. tumidus* are highly capable of $^{5+}$BMAA accumulation or more accurately sequestration, significantly more so than their size counterparts *C. javanicus* and *A. cygnea*, respectively. Whether or not this trait is due to a higher efficiency of BMAA metabolism, and which metabolic pathways are involved requires further investigation.

9.4 CONCLUSION

Data from this study constitutes the first knowledge on the metabolism of BMAA by freshwater mussels. No significant evidence was found to support the catabolism of $^{5+}$BMAA by *D. polymorpha, C. javanicus, A. cygnea* or *U. tumidus*. However, data did suggest reversible covalent modification of $^{5+}$BMAA. Whether different mussels have varying capacities for BMAA metabolism via covalent modification still requires further investigation. This information impacts significantly on the assessment of the human

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exposure risk to environmental BMAA, since this neurotoxin may be present in freshwater mussels, and possibly in other aquatic invertebrates in a non-BMAA form, which can readily be transformed into the neurotoxic amino acid. More importantly, it is conceivable that such a reversal could also occur in other organisms following ingestion or accumulation of BMAA in its modified form. The exact mechanism of BMAA metabolism remains unknown and further investigation is required to better understand this process.

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CHAPTER 10: FINAL DISCUSSION AND CONCLUSION

The BMAA-cyanobacteria hypothesis has received extensive criticism and the biological origin of BMAA and quantitative analysis of this amino acid have been controversial. To address these controversies, current BMAA analytical methods were evaluated and the most accurate and reproducible method for BMAA quantification was selected. LC-MS analysis of propyl chloroformate derivatised amino acids using EZ:faast™ is not only the most sensitive method, but also the most reproducible, accurate and precise BMAA quantification method currently in use. It is unique in that it ensures complete chromatographic separation of BMAA and its isomers 2,4-DAB and AEG in a relatively short run time, and when used in conjunction with triple quadrupole MS detection provides an extremely robust platform for BMAA quantification in complex matrices.

The cyanobacterial origin of BMAA was confirmed by showing the production of isotopically labelled BMAA from a labelled starting substrate. Investigation of the biosynthesis of BMAA by cyanobacteria also revealed that this amino acid is produced under nitrogen deprivation and that its production is closely associated with the nitrogen status of the cell as well as the nitrogen history of the culture. The fact that the presence of BMAA in cyanobacteria is modulated by specific culture conditions revealed that the inability of many investigators to detect BMAA in cyanobacteria was not necessarily solely an analytical problem. Considering that most laboratory cyanobacterial cultures are maintained under excess combined nitrogen, these conditions would not be favourable for BMAA production. Additionally, the modulation of BMAA by combined nitrogen availability is not limited to laboratory cultures and was also demonstrated in a natural environment. The presence of BMAA in the environment is therefore also highly variable, which could explain why many studies reported negative results for BMAA in various other environmental samples.

BMAA is readily metabolised in plants and in freshwater mussels. In the aquatic macrophyte, *C. demersum,* BMAA is covalently modified and is compartmentalised, presumably as part of the plant's detoxification processes. In mussels, the amino acid is covalently modified and sequestered inside the animal in a non-BMAA form.

This modification can however be reversed, and BMAA can be excreted back into the environment. The presence and persistence of BMAA in the environment is therefore highly variable as the production of the amino acid is modulated by very specific conditions, and BMAA is readily metabolised.

In cyanobacteria, cellular states that are characteristic of those that occur under nitrogen-limited conditions are induced by BMAA even in the presence of nitrogen. This strongly suggests that BMAA is specifically produced to serve in a particular nitrogen-controlled regulatory function, and that the production of the amino acid is not simply the result of metabolism associated with nitrogen deprivation. To support this hypothesis, consider the stationary phase-specific effect of BMAA on *ntc*A expression (chapter 5). Expression of *ntc*A requires modulation by the sigma factor, SigC, which although expressed at constant levels irrespective of cellular nitrogen status, is only active as a response regulator under nitrogen deprivation, specifically in stationary phase. Based on the almost constant cellular presence of this sigma factor and its growth phase specific modulation, some have suggested that SigC is specifically activated during stationary-phase (Imamura and Asayama, 2009). In the presence of BMAA, SigC causes increased *ntc*A expression in stationary phase, even in the presence of nitrogen, which suggests that BMAA may be a regulator of SigC activity. However, BMAA did not induce SigC regulated gene expression in exponential phase, which means that either an additional factor is required for BMAA-modulated SigC activation or BMAA can only induce SigC activity in stationary phase. Alternatively, BMAA or a stationary phase-specific BMAA metabolite may modulate the activity of NtcA, enhancing the autoregulated expression of this response regulator. Also, BMAA or a BMAA-metabolite may modulate the expression of *ntc*A indirectly via the modulation of PII gene (*gnl*B) expression or PII activation. The apparent stationary-specific effect of BMAA does not necessarily imply that BMAA modulates the regulatory functions of stationary-phase specific regulatory element(s), instead, the phase specific nature of BMAA regulation may be due to growth-phase specific metabolism of BMAA and the production of a regulatory metabolite.

In cyanobacteria, BMAA induces chlorosis, quiescence and *ntc*A expression, cellular states or responses that are modulated by the two main nitrogen controlled response regulators, PII and NtcA. The activities of these proteins are regulated by α-ketoglutarate levels. Considering that BMAA is a substrate for GOGAT, the consumption of BMAA by GOGAT would consequently reduce α-ketoglutarate levels, which during conventional nitrogen controlled mechanisms would result in the down regulation of PII and NtcA activity, and consequently, reduced *ntc*A expression. However, this was not observed, instead, enhanced expression of *ntc*A was shown, suggesting that the product of BMAA amination of α-ketoglutarate, the BMAA keto acid, may be a potent activator of response regulators that effect *ntc*A expression. An investigation into the effect of BMAA on PII and NtcA gene expression in the absence and presence of functional GOGAT would therefore be of interest.

The link between BMAA-induced cellular responses and stationary phase may also be explained by considering the role of GOGAT in BMAA metabolism. Two types of glutamate synthases have been identified in *Synechocystis* PCC6803; Fd-GOGAT, which is dependent on reduced ferrodoxin and NADH-GOGAT, which is dependent on the reducing power of NADH (Luque and Forchhammer, 2008). Fd-GOGAT activity requires the presence of α-ketoglutarate and reduced ferrodoxin, and appears to be the dominant functional GOGAT under conditions of high photosynthetic activity and carbon fixation rates. However, even in the absence of sufficient photoreducing power Fd-GOGAT is able to catalyse nitrogen assimilation at a level sufficient to maintain the cellular carbon-nitrogen balance. NADH-GOGAT does not appear to be essential in cyanobacteria, but does function in nitrogen assimilation when the nitrogen demand of the cell is low (Okuhara *et al.,* 1999). Based on this the following hypothesis was formulated: BMAA is present in cyanobacteria in constant equilibrium between BMAA, its β-*N*-carboxy derivative and the cyclic form of the β-*N*-carboxy derivative. Only when cellular growth slows down as a result of nitrogen limitation and NADH-GOGAT activity becomes dominant, is BMAA deaminated via transamination with α-ketoglutarate, to produce the BMAA keto acid. It is then the BMAA keto acid that acts as a response regulator, modulating the activity of other nitrogen controlled response regulators, and specifically the sigma factor SigC, the signal transduction protein PII, or the DNA binding

protein NtcA. Evidence supporting the hypothesis that it is the BMAA keto acid rather than BMAA that is responsible for regulatory effects is given by the apparent stationary phase-specific activation of regulatory element(s) by BMAA. If BMAA were responsible for the activation of SigC, or another response regulator, then the associated responses would also be observed in the exponential growth phase. However, the response was only observed in stationary phase, which suggests that stationary-phase specific metabolism of BMAA, such as deamination by NADH-GOGAT to yield the BMAA keto acid, is required for the BMAA regulatory function.

Following nitrogen deprivation, the presence of ammonia rapidly removes free cellular BMAA and reverses BMAA-induced cellular responses. Under nitrogen-replete conditions Fd-GOGAT activity becomes dominant, and consequently the production of the BMAA keto acid is reduced and the associated regulatory effects on nitrogen controlled responses cease. It is postulated that under nitrogen-replete conditions the equilibrium between BMAA, its β-*N*-carboxy derivative and the cyclic form of the β-*N*-carboxy derivative may be shifted toward the formation of cyclic β-*N*-carboxy BMAA via the consumption of this BMAA derivative through chemical modification. The metabolism of the cyclic β-*N*-carboxy derivative may account for the disappearance of free BMAA following the addition of ammonia to nitrogen-limited conditions. However, the metabolism of this cyclic β-*N*-carboxy derivative and its biological significance in cyanobacteria is currently unknown and requires further investigation.

Although the metabolism of BMAA and its associated biological function in cyanobacteria still requires further investigation, this work has identified a specific pathway that functions in BMAA metabolism and possibly also in its biological role as a regulatory element. Furthermore, BMAA metabolism and function is not only dependent on nitrogen availability, but also appears to be modulated by growth phase. This information guides future work to investigate the role of GOGAT and growth phase in BMAA metabolism, and, to verify the regulatory activity of the BMAA keto acid.

The observed metabolism of BMAA in a model plant and in mussels is not unusual or unexpected. The observed modification and sequestration of BMAA in *C. demersum* and

the similar processes observed in mussels corresponds with detoxification processes. However, in the light of the risk of human exposure to neurotoxic BMAA, these data raise some concerns. The modification of BMAA means that even though BMAA may be in the environment it will go undetected by routine analytical methods. The consequence may be the underestimation of environmental BMAA concentrations, which in turn would diminish the perceived risk of human BMAA exposure. Furthermore, as observed in mussels, the modification of BMAA can be reversed, which implies that even though BMAA is not detected in an animal or food stuff, the BMAA-derivative could, following ingestion by humans, be metabolised to yield the neurotoxic amino acid. It is therefore of key interest to identify the possible products of environmental BMAA metabolism and to determine whether such products can yield BMAA following metabolism in humans. Identification of BMAA metabolites could also serve as an indicator of the environmental presence of BMAA and could increase the accuracy of the evaluation of the human BMAA exposure risk.

BMAA is reactive and is readily metabolised and consequently the environmental fate of BMAA is determined by its metabolism in cyanobacteria and non-producing organisms. The absence of BMAA catabolism and its covalent modification in model organisms implies that BMAA can persist in the environment as BMAA or as a BMAA derivative. This impacts significantly on the assessment of the risk of human BMAA exposure. An accurate assessment and understanding of the associated risks of human exposure to BMAA and its derivatives require the characterisation of these BMAA metabolites and an investigation into their metabolism in humans.

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APPENDIX A

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Distinguishing the cyanobacterial neurotoxin β-*N*-methylamino-L-alanine (BMAA) from its structural isomer 2,4-diaminobutyric acid (2,4-DAB)

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ABSTRACT

The cyanobacterial neurotoxin β-*N*-methylamino-L-alanine (BMAA) has been associated with certain forms of progressive neurodegenerative disease, including sporadic Amyotrophic Lateral Sclerosis and Alzheimer's disease. Reports of BMAA in cyanobacterial blooms from lakes, reservoirs, and other water resources continue to be made by investigators in a variety of laboratories. Recently it was suggested that during analysis BMAA may be confused with its structural isomer 2,4-diaminobutyric acid (2,4-DAB), or that current detection methods may mistake other compounds for BMAA. We here review the evidence that BMAA can be consistently and reliably separated from 2,4-DAB during reversed-phase HPLC, and that BMAA can be confidently distinguished from 2,4-DAB during triple quadrupole LC-MS/MS analysis by i) different retention times, ii) diagnostic product ions resulting from collision-induced dissociation, and iii) consistent ratios between selected reaction monitoring (SRM) transitions. Furthermore, underivatized BMAA can be separated from 2,4-DAB with an amino acid analyzer with post-column visualization using ninhydrin. Other compounds that may be theoretically confused with BMAA during chloroformate derivatization during GC analysis are distinguished due to their different retention times.

1. Introduction

There has been renewed interest in β-*N*-methylamino-L-alanine (BMAA), a neurotoxic non-protein amino acid produced by cyanobacteria because of its possible linkage to progressive neurodegenerative illness, including sporadic amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) (Banack and Cox, 2003a; Cox et al., 2003, 2005, 2009; Ince and Codd, 2005; Papapetropoulos, 2007; Esterhuizen and Downing, 2008; Metcalf et al., 2008; Bradley and Mash, 2009; Pablo et al., 2009; Purdie et al., 2009a,b). Central to the discussion has been the detection of BMAA in complex biological matrices.

Although detection of BMAA as a purified compound or in relatively high concentrations, such as in cycad seeds is straightforward, detection of endogenous BMAA in complex organic matrices including brain tissue or cyanobacteria, particularly at low concentrations, is much more difficult (Banack et al., 2007). This was exemplified by Krüger et al. (2010) who reported an approximate 10-fold higher detection limit for BMAA and 2,4-DAB in cyanobacteria compared to detection limits achieved in cycad seeds. Furthermore, the lack of widely available reference

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materials complicates comparisons between different laboratories. Since different investigators using different methods have analyzed different materials, it is difficult to determine the comparability of differing results.

Rosén and Hellenäs (2008) suggested that what others identified in cyanobacterial extracts as BMAA is in fact DAB. Misidentification of BMAA with its structural isomer 2,4-diaminobutyric acid (2,4-DAB), also known as α-γ-diaminobutyric acid (DABA), 2,4-diaminobutanoic acid, and γ-aminobutyrine could be presumed in case of insufficient chromatographic resolution of BMAA and 2,4-DAB or if a single quadrupole MS detection was used without possibility of subsequent fragmentation. Krüger et al. (2010) used a triple quadrupole LC-MS/MS system detecting potentially unique product ions of BMAA and 2,4-DAB to distinguish these structural isomers. However, by using at least two different validated and optimized liquid chromatography methods, BMAA and 2,4-DAB have consistently been separated and are easily distinguished from one another (e.g. Banack and Cox, 2003b; Banack et al., 2007; Johnson et al., 2008).

Further importance is attached to the issues of analysis and origin due to the widespread environmental occurrence of cyanobacteria and the possible role of BMAA as an environmental toxin and potential contributor to human neurodegenerative illness (Weiss et al., 1989; Lobner et al., 2007; Rao et al., 2006; Liu et al., 2009). Here, we review the evidence for the production of BMAA by cyanobacteria, consider previous results that distinguish BMAA from 2,4-DAB, and propose a possible path to resolve such discrepancies between different laboratories in the future. If BMAA is indeed a potential trigger for ALS and Alzheimer's disease, it is important for water quality managers and health authorities to have access to reliable methods for the detection and quantification of BMAA in cyanobacterial blooms. These methods need to be validated, widely accepted, and repeatable by laboratories throughout the world.

2. BMAA and its isomer 2,4-DAB

2.1. β-N-methylamino-L-alanine, BMAA

BMAA was first isolated by Vega and Bell (1967) from seeds of *Cycas micronesica* [Cycadaceae], a gymnosperm used by the indigenous Chamorro people of Guam to manufacture flour used in tortillas, dumplings, and soups (Vega and Bell, 1967; Banack et al., 2006). Marjorie Whiting of the Burns School of Medicine, at the University of Hawaii, noting superficial resemblances between the signs and symptoms of the Chamorro disease—now called Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex (ALS/PDC), and lathyrism, wondered if β-*N*-oxalyl-L-α,β-diaminopropionic acid (BOAA), which had been implicated in lathyrism (Rao et al., 1964; Ross and Spencer, 2004), occurred in cycad seeds. Using high-voltage electrophoresis, Arthur Bell and Armando Vega isolated a new non-protein amino acid which is now known as BMAA. Subsequent studies by Nunn and colleagues showed BMAA to be neurotoxic in chicks (Polsky et al., 1972). Over the next four decades, BMAA was tested in a variety of *in vivo* systems (Vega et al., 1968; Nunn and Ponnusamy, 2009), some of which suggested neurotoxicity, while others indicated no neurotoxic effects (see review by Karamyan and Speth, 2008). Attention focused on the work of Spencer et al. (1987) who found that administration of BMAA to macaque monkeys resulted in neurological deficits. The effects, however, were acute and did not result in progressive neurodegeneration (Spencer et al., 1987). Duncan et al. (1990) questioned these results arguing that to achieve a similar dose, a Chamorro villager would have to ingest hundreds of kilograms of cycad flour (Duncan et al., 1988, 1990). Absent from this discussion, however, was a previous finding that cycad flour supernatant, after acid hydrolysis, released even more BMAA than was originally detected in the free amino acid fraction (Polsky et al., 1972). It is now known that 10–50 times the amount of BMAA occurs in the protein-bound fraction of cycad flour, compared to the free fraction (Murch et al., 2004a; Cheng and Banack, 2009). This protein-associated BMAA pool was not apparently considered in earlier assessments (Duncan et al., 1988, 1990) of the toxicological significance of Spencer's findings (Cheng and Banack, 2009).

In 2003, it was discovered that cyanobacteria of the genus *Nostoc*, which live as endosymbionts in specialized roots of cycads, produce BMAA (Cox et al., 2003). In 2005, diverse taxa of cyanobacteria, including free-living and symbiotic forms were found to produce BMAA (Cox et al., 2005). BMAA was subsequently detected in cyanobacterial blooms and laboratory isolates from marine and freshwater sources from localities as disparate as Hawaii, the United Kingdom, South Africa, Sweden and the Netherlands (Banack et al., 2007; Esterhuizen and Downing, 2008; Metcalf et al., 2008; Lurling et al., 2008; Bidigare et al., 2009; Faassen et al., 2009; Spáčil et al., 2010). At first, analytical systems such as reversed-phase HPLC separation with fluorescence detection, with peak verification via single quadrupole LC-MS, thin-layer chromatography, and GC separation with single quadrupole MS detection were used. The presence of BMAA in cyanobacteria has now been confirmed with derivatized and underivatized BMAA and using triple quadrupole LC-MS/MS. Derivatization to protect the primary and secondary amino groups is accomplished with either AQC (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate) or chloroformate, and makes the molecule easier to detect and distinguish from structurally similar compounds by increasing its molecular weight. BMAA has also been detected in cyanobacterial samples using a Hitachi amino acid analyzer in which underivatized BMAA is separated but then visualized post-column using ninhydrin (Banack et al., 2007; Cox et al., 2009; Craighead et al., 2009), in LC-MS using reversed-phase liquid chromatography (Bidigare et al., 2009), and with HILIC LC-MS/MS (Faassen et al., 2009).

2.2. Diaminobutyric acid, 2,4-DAB

There are a number of structural isomers of BMAA, but the most relevant for this discussion is 2,4-DAB (Fig. 1). 2,4-diaminobutyric acid (2,4-DAB) is a non-protein amino acid widely found in prokaryotes and eukaryotes. It was first identified as a metabolic product of bacteria found in the antibiotics polymyxin A, circulin, and colistin (Catch

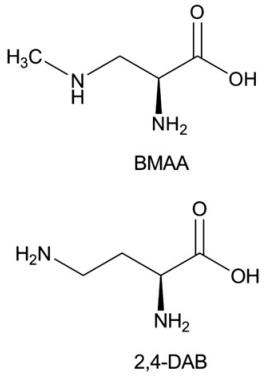

Fig. 1. Chemical structures of β–*N*–methylamino-L-alanine (BMAA), 2,4-diaminobutyric acid (2,4-DAB).

and Jones, 1948; Peterson and Reineke, 1949; Oda and Ueda, 1954). Subsequent research identified that 2,4-DAB is inserted into the peptide linkages of these naturally occurring families of antibiotics (Ressler et al., 1961). Komura and Kurahashi (1979) suggest that 2,4-DAB is attached to other amino acids by an enzyme which binds 2,4-DAB, leucine, and threonine together into a thioester and that the full synthesis of polymyxin is the result of a multi-enzyme thiotemplate mechanism. Fatty acids conjugate to the 2-amino group of 2,4-DAB which may be a "prerequisite for the initial peptide bond formation and subsequent elongation of the chain" (Komura and Kurahashi, 1979, p.1020). It is interesting to note that cyanobacteria also contain multi-enzyme complexes which are responsible for producing peptides which incorporate other non-protein amino acids (Arment and Carmichael, 1996; Dittmann and Börner, 2005). 2,4-DAB has been identified as a diagnostic diamino acid in the peptidoglycan cell wall of 65 strains of actinomycetes (Perkins and Cummins, 1964; Groth et al., 1996). In the formation of peptidoglycan in this group, the 2-amino group of the D-isomer of 2,4-DAB forms a peptide bond with the carboxyl group of D-alanine (Schleifer and Kandler, 1972). The L-isomer is found in the muramic acid pentapeptide whereas the D-isomer forms the cross linkage.

Within the eukaryotes, 2,4-DAB was first identified in the rhizome of *Polygonatum multiflorum* Kunth [Convallariaceae] (Fowden and Bryant, 1958) and subsequently found in a slug, *Arion empiricorum* Fer. [Arionidae] (Ackermann and Menßen, 1960). It has subsequently been found at low concentrations in 80 out of 145 angiosperm species, including monocotyledons and dicotyledons (VanEtten and Miller, 1963). Substantial concentrations are found in legumes (Ressler et al., 1961; Bell and Tirimanna, 1965; Evans et al., 1985), particularly in the flatpea, *Lathyrus sylvestris* L. [Fabaceae] where it represents 10.3% of plant nitrogen (VanEtten and Miller, 1963). 2,4-DAB was also found in bovine brain (Nakajima et al., 1967). More recently, 2,4-DAB was isolated from cycad seeds (*Cycas angulata* R.Br., *Cycas rumphii* L. [Cycadaceae]) along with 11 other "non-protein" amino acids from nine cycad species examined (Pan et al., 1997). Banack and Cox (2003b) isolated 2,4-DAB and distinguished it from BMAA within the leaves, mature male sporangia and female sporophyll of *C. micronesica* Hill.

2,4-DAB has been identified as having "strong neurotoxic action" in the rat with 7 mmol/kg producing behaviors including hyperirritability, weakness in hind legs, tremors in the upper extremities and convulsions leading to death (Riggs et al., 1954; Ressler et al., 1961; O'Neal et al., 1968; Chen et al., 1972). Cells (e.g. tumor and liver) rapidly take up 2,4-DAB (Riggs et al., 1954; Neal and Bowery, 1977; Weitsch-Dick et al., 1978). When exposed to 2,4-DAB, the ganglion cells of the cerebellum and the cerebral cortex shrink and exhibit pyknosis (Riggs et al., 1954). O'Neal et al. (1968) described liver damage from 2,4-DAB which he suggested led to secondary brain neurotoxicity by inducing ammonia toxicity through the inhibition of the liver enzyme ornithine transcarbamylase which disrupts the urea cycle (O'Neal et al., 1968; Rasmussen et al., 1993). 2,4-DAB crosses the blood–brain barrier using the cationic amino acid transporter system y+ and causes GABA to accumulate in the brain (Chen et al., 1972; Shuter and Robins, 1974; Stoll et al., 1993). Since neurotoxicity was only observed at 1–2 μmol 2,4-DAB/g, Chen et al. (1972) attributed the toxicity to the penetration of these compounds into the brain. They proposed that 2,4-DAB toxicity resulted from decarboxylation to diaminopropane (Chen et al., 1972). 2,4-DAB was noted to be a potent inhibitor of ^{3}H-GABA uptake into Na^{+} dependent nerve terminals (Dick and Kelly, 1975). However, since the inhibition of sodium-independent GABA channels was found to be non-stereospecific, Johnston and Twitchin (1977) concluded that the neurotoxicity of 2,4-DAB was not related to the inhibition of GABA uptake but instead to interaction with postsynaptic receptors for GABA. 2,4-DAB has been tested as a possible therapeutic drug to block GABA uptake and, therefore, to protect against seizures (Taberner and Roberts, 1978; Horton et al., 1979; Strain et al., 1984; Erecińska et al., 1986; Malizia and Tunnicliff, 1987; Leal et al., 2004).

Since 2,4-DAB neuroexcitation and neurotoxicity are increased in the presence of bicarbonate (Weiss and Choi, 1988), it has been suggested that "DAB might contribute to the pathogenesis of neuronal cell loss associated with neurolathyrism or another chronic neurodegenerative condition" (Weiss et al., 1989, p. 323).

2.3. Distinguishing BMAA from its structural isomer 2,4-DAB

2,4-DAB can be distinguished from BMAA using suitable separation methods including high performance liquid chromatography with fluorescence detection (HPLC-FD) (Fig. 2), ultra performance liquid chromatography with ultra-violet detection (UPLC-UV) (Fig. 3B), UPLC with single quadrupole mass spectrometry (UPLC-MS) (Fig. 3A), triple quadrupole liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) (Figs. 4 and 5) or ultra performance liquid chromatography with single quadrupole mass spectrometry (HPLC-MS) (Fig. 6). 2,4-DAB has been used routinely as a standard control for HPLC-FD, UPLC-UV, UPLC-MS, LC-MS/MS and Hitachi amino acid analyzer analysis of BMAA to ensure adequate separation

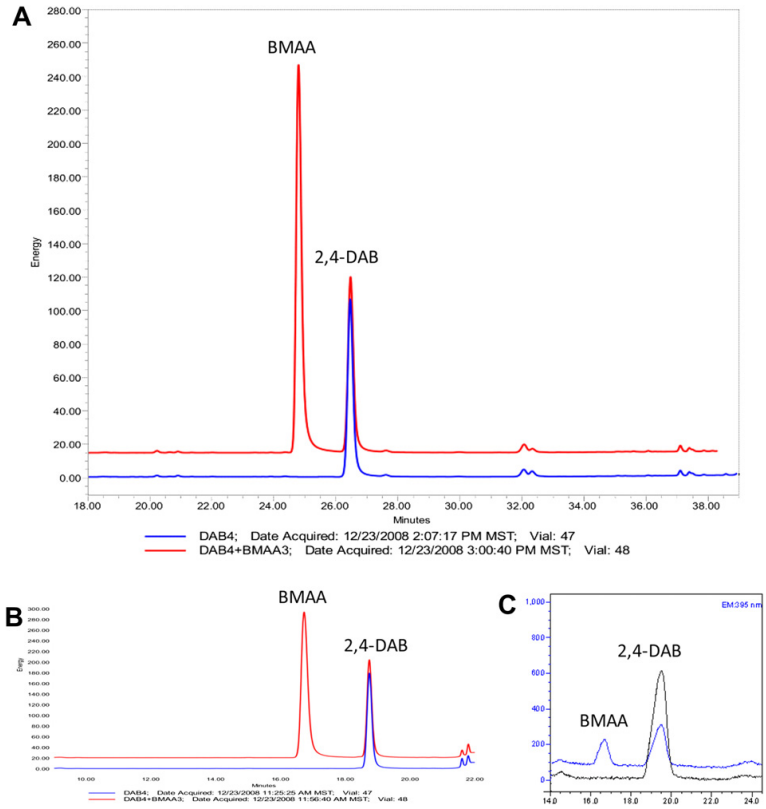

Fig. 2. A. Separation of 2,4-DAB and BMAA standards using AQC derivatization run on an HPLC-FD with a 60 min gradient (see Banack and Cox, 2003a). Red line is BMAA plus 2,4-DAB; blue line is 2,4-DAB. B. Separation of 2,4-DAB and BMAA standards using AQC derivatization run on an HPLC-FD with a 30 min gradient (see Pablo et al., 2009). Red line is BMAA plus 2,4-DAB; blue line is 2,4-DAB. C. HPLC-FD chromatogram, 45 min gradient. Black: 1 μg/ml 2,4-DAB standard, Blue: 0.5 μg/ml BMAA standard and 0.5 μg/ml 2,4-DAB following Purdie et al. (2009a,b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and correct identification based on product ions in the triple quadrupole LC-MS/MS (Banack and Cox, 2003b; Banack et al., 2007; Johnson et al., 2008). For example, Banack et al. (2007, p. 194) state that 2,4-DAB was used as a control: "a blank of L-2,4-diamino-*n*-butyric acid which has a molecular weight of 118.13, equivalent to BMAA but with a different structure, retention time, and product ions." Apart from previously mentioned LC methods for the analysis of BMAA, GC methods (Guo et al., 2007; Esterhuizen and Downing, 2008; Snyder et al., 2009), and a nuclear magnetic resonance (^{1}H NMR) method (Moura et al., 2009) have been recently published. However it seems that LC and GC methods are the most suitable for BMAA in terms of sensitivity, dynamic range, and selectivity and therefore these will be discussed in more detail.

2.3.1. Methods for distinguishing BMAA from 2,4-DAB using liquid chromatography

In principle, both direct and indirect analysis of BMAA and 2,4-DAB are possible depending on the LC stationary phase employed. Underivatized compounds can be successfully separated using two different methods: 1) a Li^+-based cation exchange resin column followed by post-column ninhydrin reaction and aplanatic concave diffraction grating spectrophotometry (wavelength; 570 nm, 440 nm) automated in a Hitachi amino acid analyzer (Fig. 7) and, 2) using hydrophilic interaction liquid chromatography (HILIC) followed by MS or MS/MS detection. Likewise, less polar derivatives formed after reaction with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) are effectively separated by conventional reversed-phase LC (RPLC) and can be detected using UV, FD, MS and MS/MS (Figs. 2–5). Similarly, chloroformate derivatives of BMAA and 2,4-DAB are also easily separated by liquid chromatography, are relatively stable and are detectable by MS (Fig. 6).

To date most, but not all (e.g. Faassen et al., 2009), applications of HILIC procedures have not detected BMAA in cyanobacterial samples (Table 1). This result may be explained by several contributing factors. First, since HILIC is a newer technique than RPLC, the basic chemistry in RPLC

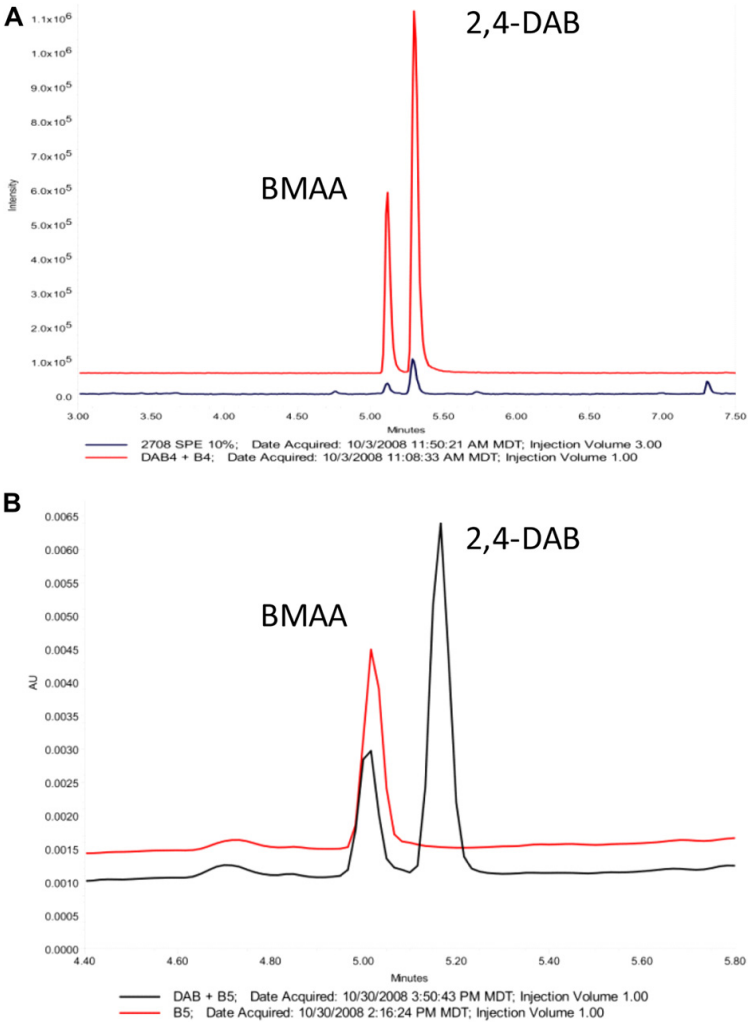

Fig. 3. A. Separation of 2,4-DAB and BMAA standards using AQC derivatization run on an UPLC with single quadrupole mass spectrometry detection (see Banack et al., 2007). Red line shows BMAA plus 2,4-DAB; blue line shows BMAA and 2,4-DAB concurrently in a desert cyanobacterial crust from Qatar (See Cox et al., 2009). B. Separation of 2,4-DAB and BMAA standards using AQC derivatization run on an UPLC with UV detection (see Banack et al., 2007). Red line shows BMAA; black line shows BMAA and 2,4-DAB standards mixed together). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

is better understood. Generally the HILIC columns have lower performance than RPLC columns as noted by a recent review (Ikegami et al., 2008). Specifically, the HILIC resulted in lower column efficiency, expressed by broader chromatographic peaks, and/or insufficient resolution (Ikegami et al., 2008; Krüger et al., 2010 Fig. 2A). Furthermore, lower column efficiency might cause significant matrix effects resulting in shifts in retention time or detection signal suppression. A second factor explaining difficulties of the HILIC to detect BMAA, is related to efficiency in the electrospray ion source (ESI). Analytes have to be charged in the ESI source before MS detection, therefore, ionization efficiency significantly affects detection sensitivity. From the ESI fundamentals, desorption of charged molecules occurs from the surface, meaning that sensitivity is higher for compounds with a greater concentration at the surface of the droplet, thus the more lipophilic ones rather than the hydrophilic compounds in the interior of the droplet (Hoffmann and de Strobant, 2007). AQC derivatives are much more lipophilic compared to underivatized BMAA and 2,4-DAB molecules and also have higher molecular weight, which ideally suits triple quadrupole tandem MS

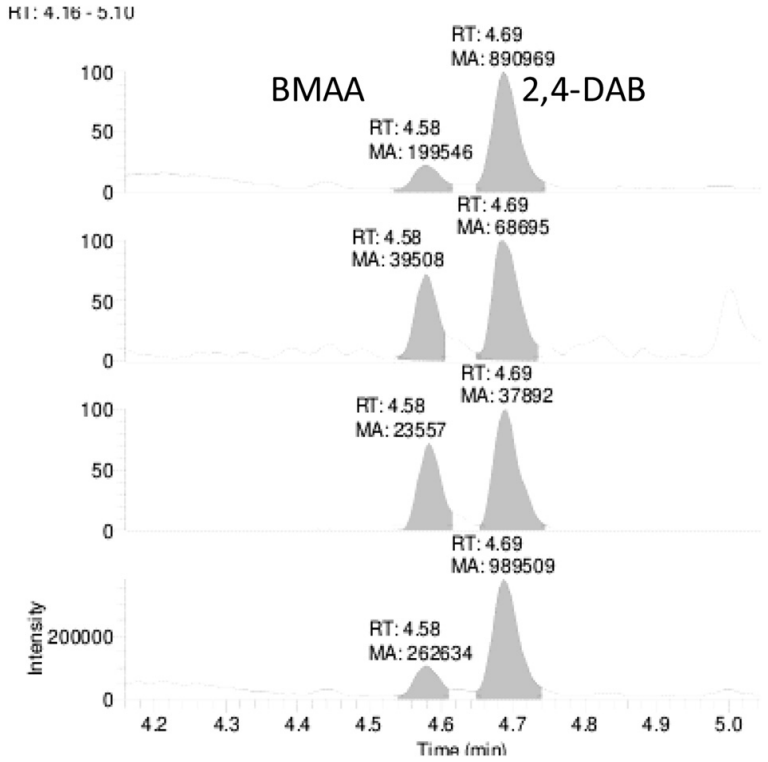

Fig. 4. BMAA and 2,4-DAB separated in a triple quadrupole mass spectrometer within a desert crust sample from Qatar (see Cox et al., 2009). Retention time (rt) and product ion ratios formed from collision-induced dissociation clearly differentiate BMAA (rt = 4.58 min; ratios 11.86, 100, and 21.76 for ions 119, 171, and 289 respectively) from 2,4-DAB (rt = 4.69 min; ratios 4.40, 100, and 8.14 for ions 119, 171, and 289 respectively) when compared with an authenticated standard.

detection. Finally, the reaction of AQC with amino acids was described almost two decades ago (Cohen and Michaud, 1993) and that of amino acids with chloroformate almost three decades ago (Einarsson et al., 1983) and due to reliability and robustness these derivatization methods continue to be routinely used today in critical medical settings.

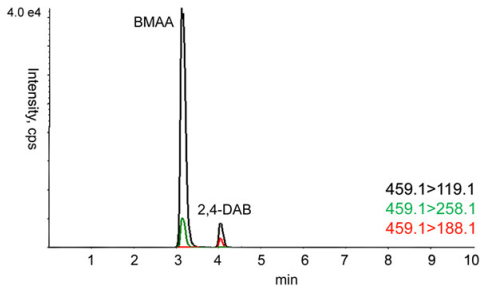

Fig. 5. Typical mass spectrum acquired using a triple quadrupole mass spectrometer and liquid chromatography (see Spácil et al., 2010). Standards of derivatized BMAA (0.35 μg/L) at the retention time 3.1 min and 2,4-DAB (0.35 μg/L) at the retention time 4.0 min. Color legend indicates Selected Reaction Monitoring (SRM) transition used for confident identification of BMAA and 2,4-DAB: 459.1 > 119.1 (non-specific detection of BMAA and 2,4-DAB); 459.1 > 258.1 (specific detection of BMAA); 459.1 > 188.1 (specific detection of 2,4-DAB).

Rosén and Hellenäs (2008) using a HILIC method tested 30 laboratory-raised strains of cyanobacteria and four cyanobacterial bloom samples from the Baltic Sea. They detected low concentrations of BMAA which they could not confirm: "in two samples, traces [<4 μg g^{-1}, total BMAA] were detected at m/z 119 > 102, however, at this low level other transitions could not be used to confirm the identity of BMAA" (p. 1788). If the transitions at low m/z are used in tandem mass spectrometry based on Selected Reaction Monitoring (SRM), the limit of detection (LOD) might be less sensitive due to higher noise rate. Hence the SRM of product ions originating from underivatized BMAA is less favorable than the SRM detection of derivatized BMAA, which has substantially higher molecular weight and less inherent background noise. They identified 2,4-DAB in a single sample and concluded that the origin of 2,4-DAB in their analyzed samples was unclear. Rosén and Hellenäs (2008, p. 1788) state that "retention time is thus the only criterion for identification" of BMAA in previous papers. However, at least two distinct analytical methods were used together to detect BMAA in the early papers published

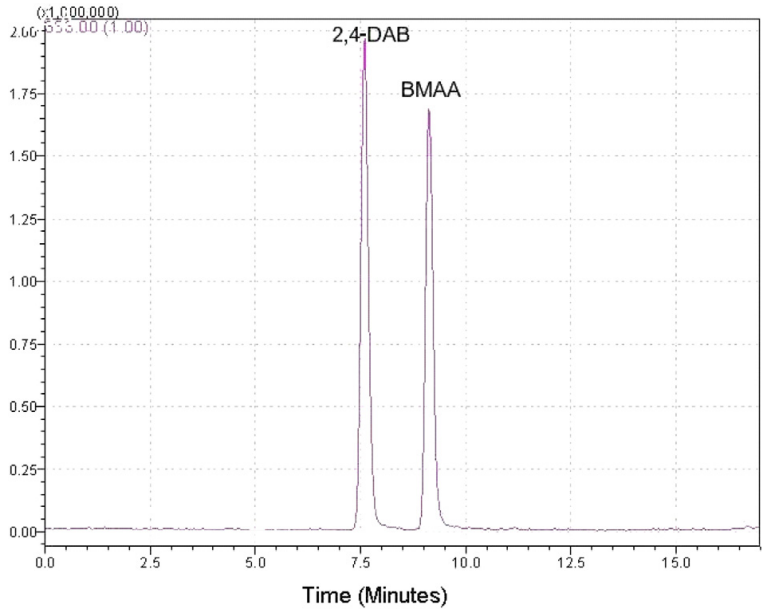

Fig. 6. Shimadzu 2010EV LC-MS single quadrupole detection of separated propylchloroformate-derivatized BMAA and 2,4-DAB using a 250 × 2.0 mm EZ:faast™ AAA LC column (Phenomenex).

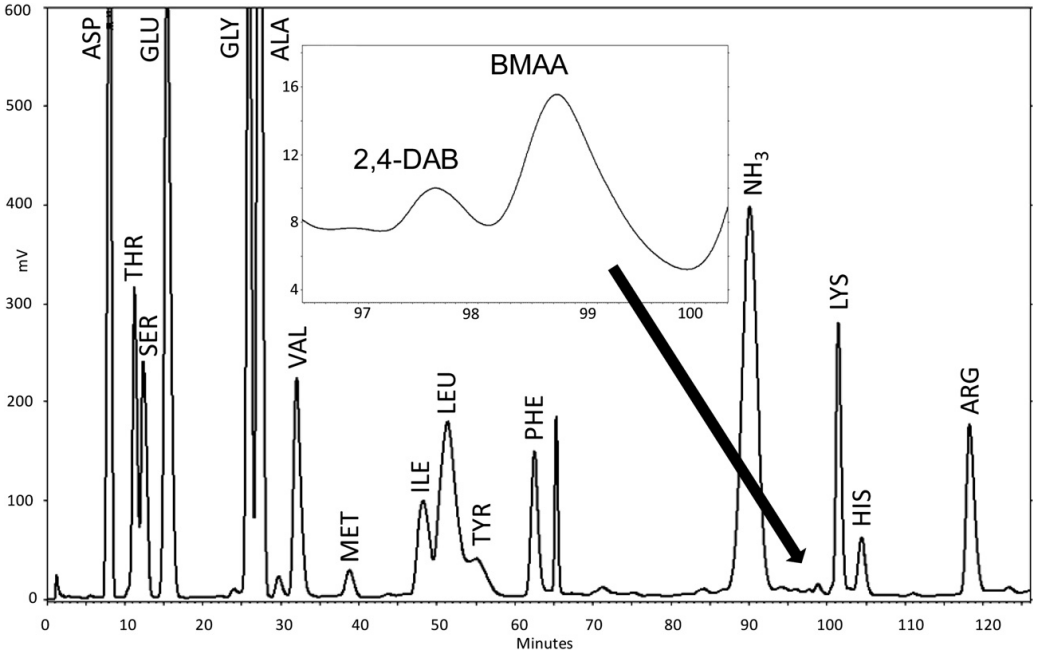

Fig. 7. Hitachi Amino Acid Analyzer separation of 2,4-DAB and BMAA in an underivatized endophytic *Nostoc* sample cultured from a Guam cycad.

Table 1
Detection of BMAA in cyanobacterial samples using reversed-phase LC, HILIC, and GC methods.

Was BMAA detected?	Samples with BMAA	Method used	LOD (pmol/inj)	Pre-column derivative	Reference
No	0/2	HILIC-MS	4	No	Kubo et al. (2008)
No	0/36	HILIC-MS/MS	#	No	Rosén and Hellenäs (2008)
No	0/30	HILIC-MS/MS	#	No	Krüger et al., (2010)
No	0/3	HILIC-MS/MS	0.017	No	Li et al. (2010)
Yes	4/8	HILIC-MS/MS	0.34	No	Faassen et al. (2009)
Yes	1/1	Ion-exchange	42^	No	Banack et al. (2007)
Yes	26/27	GC–MS	4.2	ECF	Esterhuizen and Downing (2008)
Yes	1/1	RPLC-MS/MS	0.07^	AQC	Banack et al. (2007)
Yes	1/1	RPLC-MS/MS	0.17^	AQC	Johnson et al. (2008)
Yes	12/12	RPLC-MS/MS	0.07^	AQC	Metcalf et al. (2008)
Yes	1/3	RPLC-MS/MS	0.07^	AQC	Li et al. (2010)
Yes	2/2	RPLC-MS/MS	0.023	AQC	Spáčil et al. (2010)
Yes	4/4	RPLC-MS	^	AQC	Cox et al. (2003)
Yes	2/2	RPLC-MS	^	AQC	Murch et al. (2004a)
Yes	37/41	RPLC-MS	^	AQC	Cox et al. (2005)
Yes	1/1	RPLC-MS	0.17^	AQC	Banack et al. (2007)
Yes	21/21	RPLC-MS	0.17^	AQC	Johnson et al. (2008)
Yes	1/1	RPLC-MS	^	No	Bidigare et al. (2009)
Yes	4/5	RPLC-FD	130	AQC	Banack and Cox (2003b)
Yes	4/4	RPLC-FD	130*	AQC	Cox et al. (2003)
Yes	2/2	RPLC-FD	130*	AQC	Murch et al. (2004a,b)
Yes	37/41	RPLC-FD	65	AQC	Cox et al. (2005)
Yes	1/1	RPLC-FD	130*	AQC	Banack et al. (2007)
Yes	21/21	RPLC-FD	65	AQC	Johnson et al. (2008)
Yes	12/12	RPLC-FD	65	AQC	Metcalf et al. (2008)
Yes	1/1	RPLC-FD	0.12	AQC	Eriksson et al. (2009)
Yes	3/3	RPLC-FD	5	AQC	Bidigare et al. (2009)
Yes	1/1	RPLC-UV	0.1	AQC	Banack et al. (2007)
Yes	21/21	RPLC-UV	0.1^	AQC	Johnson et al. (2008)

LOD was expressed as μg/g; ^LOD provided by authors; *LOD from Banack and Cox (2003a,b).

on BMAA in cyanobacteria (Banack and Cox, 2003a,b; Cox et al., 2003, 2005, 2009; Murch et al., 2004a,b; Banack et al., 2006, 2007; Johnson et al., 2008; Cheng and Banack, 2009). Rosén and Hellenäs (2008, p. 1788) further state "difficulties in obtaining quantitative derivatization for BMAA by the method have been reported" referring to Eriksson et al. (2009). However, Eriksson et al. (2009) used different columns and analytical equipment to develop a new method; subsequently, their laboratory at Stockholm University is routinely detecting and quantifying BMAA in cyanobacterial samples from the Baltic Sea (Jonasson et al., 2010). The inference of Rosén and Hellenäs (2008) is that 2,4-DAB has routinely been mistaken for BMAA. However, since 2,4-DAB and BMAA have different retention times, different product ion ratios, and in all HPLC, UPLC, LC-MS/MS platforms are clearly distinguished and since 2,4-DAB is routinely used as a control in sample queues, there is no confusion in distinguishing these isomers beginning with the first paper in 2003 (Banack and Cox, 2003b).

Krüger et al. (2010) also used a HILIC method and did not detect BMAA in cyanobacterial samples. Interestingly, Krüger et al. (2010) detected BMAA in two commercial samples of *Spirulina* at 57.9 and 26.3 μg/g respectively at *m*/*z* 341.3 amu using 9-fluorenylmethyl chloroformate derivatization (FMOC), but did not detect BMAA in these samples using HILIC (Krüger et al., 2010, p. 554).

Krüger et al. (2010) state that BMAA was detected by Banack et al. (2007) in cyanobacteria solely by "the determination of the neurotoxic amino acid after derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC)." However, Banack et al. (2007) detected BMAA in the *Nostoc* strain CMMED-1 using a method involving underivatized amino acids directly injected into a Hitachi amino acid analyzer (AAA), in which no AQC was involved (Banack et al., 2007, p. 185, Fig. 5). As noted in Banack et al. (2007, p. 192), sample batches for the AAA included "L-2,4-diamino-*n*-butyric acid, which has a molecular weight of 118.13 equivalent to BMAA," and complete separation between underivatized BMAA and underivatized 2,4-DAB was achieved using this method (Fig. 3). Krüger et al. (2010) further state that although Banack et al. (2007) "broached the issue of DAB, tandem mass spectrometry confirmation was performed using product ions *m*/*z* 289 $[M + AQC + H]^+$, 171 $[AQC]^+$ and 119 amu $[M + H]^+$ of the precursor ion *m*/*z* 459 $[M+2AQC + H]^+$ which did not allow the discrimination of DAB." However, Banack et al. (2007, p. 194) stated (referring to triple quadrupole LC-MS/MS), "Sample batches included… a blank of L-2,4-diamino-*n*-butyric acid which has a molecular weight of 118.13, equivalent to BMAA but with a different structure, retention time, and product ions." As is clear from Banack et al. (2007), the different retention time and product ion ratios produced by BMAA and 2,4-DAB precisely distinguish these two isomers.

Krüger et al. (2010) also considered chloroformate derivatization a problem in that the EZ:faast™ kit creates amino acid conjugates such that several different amino acids have the same mass to charge ratios (*m*/*z*). This concern can be overcome if amino acids are separated by retention time during chromatography prior to mass

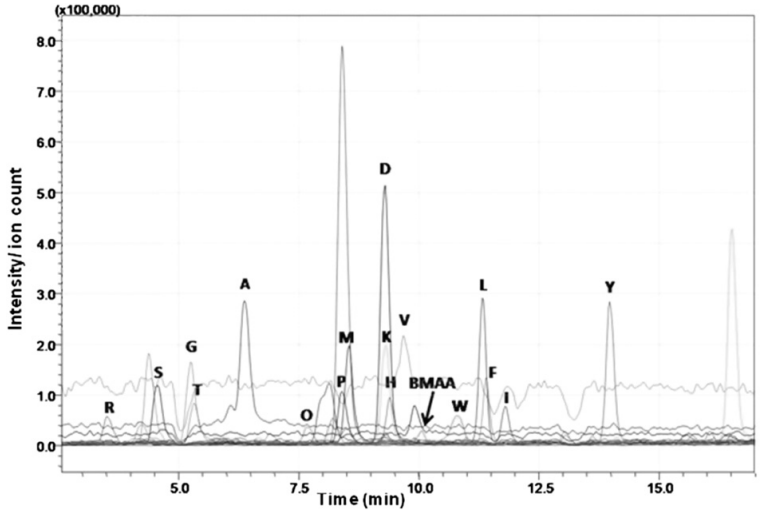

Fig. 8. Solid phase extraction recovery of BMAA from an amino acid matrix, detected using a Shimadzu 2010EV LC-MS single quadrupole for propylchloroformate-derivatized amino acids. Samples were derivatized as described by Esterhuizen and Downing (2008) using the EZ:faast™ amino acid analysis kit for LC-MS (Phenomenex). BMAA was separated from other amino acids by liquid chromatography on a commercial column (Phenomenex AAA-MS 250 × 2.0 mm amino acid analysis column) using a LC-MS 2010EV (Shimadzu). A solvent gradient was used with A: 10 mM ammonium formate in water and B: 10 mM ammonium formate in methanol (0.0 min = 68% B, 13.00 min = 83% B, 13.01 min = 68% B, 17.00 min = 68% B) at a flow rate of 0.25 ml/min and 1 µl sample injection volume. Column temperature was kept constant at 35 °C. The mass spectrometer ESI source (positive ion mode) temperature was set at 250 °C. The ion scan range was between 20 and 600 *m/z*. The detector voltage was set at 1.5 kV. The interface voltage was set at 4.5 kV and the CDL voltage at −20 V with the heating block at 200 °C. Data were analyzed using LC-MS solutions ver. 3 software. Detection by MS was as follows: Arginine (R) 303 *m/z*, Serine (S) 234 *m/z*, Glycine (G) 204 *m/z*, Threonine (T) 248 *m/z*, Alanine (A) 218 *m/z*, Ornithine (O) 347 *m/z*, Proline (P) 244 *m/z*, Methionine (M) 278 *m/z*, Lysine (K) 361 *m/z*, Aspartate (D) 304 *m/z*, Histidine (H) 370 *m/z*, Valine (V) 246 *m/z*, BMAA 333 m/z, Tryptophan (W) 333 *m/z*, Leucine (L) 260 *m/z*, Phenylalanine (F) 294 *m/z*, Isoleucine (I) 260 *m/z*, Tyrosine (Y) 396 *m/z*.

spectrometry. Using an ethylchloroformate (ECF) derivatization with gas chromatography mass spectrometry (GC-MS) detection, Esterhuizen and Downing (2008) identified BMAA (130.2 *m/z*) with a retention time of 3.95 min. Ions with similar *m/z* ratios such as alanine, sarcosine, and tryptophan all elute at different times (1.8, 1.9, and 6.85 min respectively) in this system (Nozal et al., 2004) and therefore pose no conflict to identification. Similarly, using a propylchloroformate derivatization intended for LC-MS detection, BMAA, 2,4-DAB, and tryptophan all have equal mass; $[M + H]^+ = 333$ amu (*m/z*). These analytes all separate chromatographically; without a matrix, BMAA and 2,4-DAB pure standards derivatized and injected elute 1.5 min apart (2,4-DAB rt = 7.7 min; BMAA rt = 9.2 min, Fig. 6). Within a matrix there typically is a slight shift in retention times but separation is not affected; BMAA elutes 0.7 min from tryptophan (BMAA rt = 10.1 min, tryptophan rt = 10.8 min, Fig. 8). Thus, the concern that the ions are being misidentified due to similar mass is eliminated due to the chromatographic separation prior to mass detection.

Krüger et al. (2010) did not detect either BMAA or 2,4-DAB in the South African *Synechocystis* strain J341, while Esterhuizen and Downing (2008) did detect BMAA, leading Krüger et al. (2010) to consider the possibility that Esterhuizen and Downing may have misidentified both neurotoxic isomers. It is unclear how the result of Krüger et al. (2010) negates the positive detection by Esterhuizen and Downing (2008). It appears on the surface that the same sample, the South African *Synechocystis* strain J341, was tested by both Esterhuizen and Downing (2008) and Krüger et al. (2010). However, the strain analyzed by Esterhuizen and Downing (2008) was a unique proprietary isolate of the Nelson Mandela Metropolitan University and Krüger et al. (2010) did not request this strain from the source. Since the origin and provenance of this sample is unclear, Krüger et al. may wish to retest this sample using a verified pure strain. Having different laboratories test the same material can help resolve differences. For example, both Cox et al. (2005) and Spáčil et al. (2010) detected BMAA in the same strain of *Plectonema* PCC 73110, previously known as *Leptolyngbya* PCC 73110. And, Kubo et al. (2008) for example, were unable to detect BMAA in two cyanobacterial strains using LC-MS methods. When these same strains were analyzed at the Institute for Ethnomedicine by LC-MS/MS, BMAA was similarly not detected. Starting from common ground and replicating the findings of others, by using the same methods and same samples, would provide an excellent place to begin discussions.

2.3.2. Methods for distinguishing BMAA from 2,4-DAB using gas chromatography

GC separation requires conversion of BMAA and 2,4-DAB to non-polar, volatile products, which is accomplished by reaction with ethylchloroformate (Guo et al., 2007;

Esterhuizen and Downing, 2008; Snyder et al., 2009). The detection limit achieved by Esterhuizen and Downing (2008) was 4.2 pmol on column, which is comparable with published LC methods, although some LC methods were able to provide even 200-fold higher sensitivity (see Table 1). This GC method was successfully used to detect free and/or bound BMAA in thirteen free-living freshwater cyanobacterial strains (Esterhuizen and Downing, 2008). On the other hand, in the method used by Snyder et al. (2009) the limit of detection for protein-associated BMAA was 5.0 μg/g, which might be insufficient for the detection of trace BMAA concentrations.

3. Discussion

The putative link between BMAA and some forms of progressive human neurodegenerative disease has led to the need to analyze this amino acid in cyanobacteria. In order to resolve differences between research laboratories and to determine what risk BMAA poses to human health, consensus within the scientific community should be reached. First, a comparison of methods starting with repeating the validated methods of researchers who have identified BMAA would build some common ground and act as a starting point for a comparative analysis. This comparison should include a balanced view on whether HILIC chromatography is adequate for BMAA analysis within a complex matrix and a check to ensure that the methods employed provide unambiguous determination of BMAA. The fact that BMAA was positively identified using multiple methods (including both the underivatized and derivatized amino acids) conducted on the same cyanobacterial material (Banack et al., 2007) and that multiple laboratories have also found BMAA in axenic cyanobacterial cultures and cyanobacterial blooms (Metcalf et al., 2008; Esterhuizen and Downing, 2008; Faassen et al., 2009; Spáčil et al., 2010) supports the conclusion that BMAA is widely produced by cyanobacteria.

The development of standardized reference materials available to all researchers could facilitate comparison across laboratories. Since the content of BMAA within a single strain varies during growth phase (Cox et al., 2005), it would be of interest to develop several freeze-dried standards from strains with known provenance and processed in a single batch. This would allow direct comparisons and facilitate greater communication within the research community.

Increasingly, cyanobacterial genomes are being sequenced and mined for proteins and enzymes and with a greater understanding of biosynthetic pathways, molecular techniques may be amenable to BMAA and 2,4-DAB assessment. Recently, a biosynthetic pathway for BMAA in cyanobacteria was proposed (Aráoz et al., 2010) and this may prove to be a useful additional means of determining the potential for BMAA to be produced by cyanobacteria.

Finally, due to the implications of BMAA production and its potential effects on long-term human health, further research is required in order to determine the extent of neurotoxic amino acid production by cyanobacteria and whether water- and airborne-BMAA present significant human exposure and related health risks.

4. Conclusion

BMAA and its neurotoxic structural isomer 2,4-DAB are produced by cyanobacteria. These isomers are routinely and clearly distinguished by having different retention times during chromatographic separation of underivatized BMAA (Hitachi amino acid analyzer) as well as derivatized BMAA using four distinct derivatization methods (AQC, FMOC, propylchloroformate, ECF). Even though BMAA and 2,4-DAB have the same molecular weight, they can be distinguished by triple quadrupole LC-MS/MS analysis, due to the collision-induced dissociation resulting in diagnostic product ions in consistent ratios. The detection of BMAA in cyanobacteria by seven different laboratories in five different countries (Germany, the Netherlands, the UK, South Africa, Sweden, and the US) by different investigators using different techniques shows that 1) BMAA is indeed produced by cyanobacteria, and 2) that using a standardized, validated, rapid method for detection and quantification of BMAA that is accepted internationally will be of value to scientists, health care professionals, and water resource managers in the future.

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Conflict of interest statement

The authors have no conflict of interest.

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Distinguishing the cyanobacterial neurotoxin β-*N*-methylamino-L-alanine (BMAA) from other diamino acids

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ABSTRACT

β-*N*-methylamino-L-alanine (BMAA) is produced by diverse taxa of cyanobacteria, and has been detected by many investigators who have searched for it in cyanobacterial blooms, cultures and collections. Although BMAA is distinguishable from proteinogenic amino acids and its isomer 2,4-DAB using standard chromatographic and mass spectroscopy techniques routinely used for the analysis of amino acids, we studied whether BMAA could be reliably distinguished from other diamino acids, particularly 2,6-diaminopimelic acid which has been isolated from the cell walls of many bacterial species. We used HPLC-FD, UHPLC-UV, UHPLC-MS, and triple quadrupole tandem mass spectrometry (UHPLC-MS/MS) to differentiate BMAA from the diamino acids 2,6-diaminopimelic acid, N-2(amino)ethylglycine, lysine, ornithine, 2,4-diaminosuccinic acid, homocystine, cystine, tryptophan, as well as other amino acids including asparagine, glutamine, and methionine methylsulfonium.

1. Introduction

The possible role of the cyanobacterial toxin β-*N*-methylamino-L-alanine (BMAA) in triggering neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease in vulnerable individuals has resulted in an increased interest in this unusual amino acid (Bradley and Mash, 2009). BMAA is present in environmental samples and human tissues both as a free amino acid, and as a protein-bound amino acid (Murch et al., 2004a, b). It has been hypothesized that its misincorporation into polypeptide sequences results in protein misfolding and collapse. It has also been hypothesized that misincorporation of BMAA triggers protein collapse which is linked to various tangle diseases characterized by irreversible progressive neurodegeneration (Murch et al., 2004a, b; Bradley and Mash, 2009).

Studies of laboratory animals have shown that even a low rate of misincorporation of standard protein amino acids can trigger neurodegenerative disease (Lee et al., 2006).

Misincorporation of the non-protein amino acid L-canavanine results in anomalous proteins and toxicity in herbivores which forage on *Canavalia ensiformis* [Fabaceae] (Allende and Allende, 1964; Rosenthal, 1977). Proteins with misincorporated L-Dopa have been shown to be more resistant to proteolysis and prone to cross-linking and aggregation (Rogers and Shiozawa, 2008).

BMAA is specifically toxic to motor neurons (Rao et al., 2006) as an agonist at NMDA and AMPA receptors, induces oxidative stress, and promotes accumulation of extracellular

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glutamate through its action on the cystine/glutamate antiporter (system X_c^-) (Liu et al., 2009). Furthermore, BMAA potentiates neuronal damage from other neurotoxic insults (Lobner et al., 2007). It has been hypothesized that vulnerable individuals may be at increased risk of progressive neurodegenerative illness if exposed to BMAA (Cox et al., 2003; Banack and Cox, 2003a). Given the relative ubiquity of cyanobacteria, which have successfully colonized habitats ranging from Antarctica to the deserts of the Middle East, and which often are present as blooms, mats, or scums in freshwater, estuarine, and marine waterbodies, there is a possibility of exposure to BMAA in disparate parts of the globe (Cox et al., 2005, 2009; Metcalf et al., 2008).

Due to the possible clinical implications of BMAA exposure and the wide array of compounds produced by cyanobacteria, it is important to distinguish BMAA from other similar molecules. To date BMAA has been routinely distinguished from other proteinogenic amino acids as well as its neurotoxic isomer 2,4-diaminobutyric acid (2,4-DAB) by reversed-phase HPLC methods (Banack et al., 2007, 2010) and with the most selectivity by UHPLC with triple quadrupole tandem mass spectrometry (UHPLC-MS/MS) using distinct retention times, diagnostic product ions and characteristic ratios between selective reaction monitoring transitions (Banack et al., 2010; Spáčil et al., 2010).

Another BMAA isomer, N-2(amino)ethylglycine, was analyzed along with other diamino acids and amides, which could potentially interfere with BMAA analysis. We focused on compounds that might occur in complex physiological matrices, such as 2,6-diaminopimelic acid, found in the cell walls of many bacterial species (Work and Dewey, 1953).

2. Materials and methods

2.1. Chemicals

L-BMAA was synthesized by Dr. Peter Nunn and compared to Sigma B-107 (St. Louis, MO). 2,6-diaminopimelic acid (synonym: 2,6-diaminoheptanedioic acid) from Sigma (D1377) was compared to Research Organics # 2045D (Cleveland, OH). N-2(amino)ethylglycine was from TCI America (Portland, OR); Part # A1153. L-lysine, L-ornithine monohydrochloride, L-asparagine, L-glutamine, L-cystine, L-tryptophan, L-2,4-diaminobutyric acid dihydrochloride, methanol and acetonitrile (LC-MS CHROMASOLV®) were purchased from Sigma–Aldrich (St. Louis, MO) as Part #'s L5501, O2375, A0884, G8540, C7602, T0254, 32830, 34966, and 34967 respectively. Ammonium formate (NC9841158) was from Fisher Scientific. 2,4-diaminosuccinic acid [synonym meso-A,A-diaminosuccinic acid] was purchased from Research Organics #0255D (Cleveland, OH). L-homocystine was obtained from MP Biomedicals (Solon, OH); Part # 521061580. Formic acid was purchased from Acros Organics (Geel, Belgium, distributed by Fisher Scientific, NJ, # 270480025). Proprietary compositions of UHPLC eluent A: part #186003838, UHPLC eluent B: part # 186003839, and HPLC eluent A part # 052890 were obtained from Waters Corp. (Milford, MA). Water was purchased from Fisher W6-4 Optima LC/MS for use with the LC/MS/MS or purified in-house (Direct-Q uv3, Millipore Ltd, Bedford, MA) to 18.2 MΩ quality for all other purposes. For comparison purposes, DL-methionine methylsulfonium chloride (Sigma # 64382) was analyzed.

2.2. Standard preparation

All compounds were reconstituted in 20 mM HCl except for L-homocystine and 2,4-diaminosuccinic acid which were initially dissolved in 0.5 M NaOH before being diluted with water or 20 mM HCl for analysis. Each compound was analyzed both as an acid hydrolysate prepared in a final concentration of 6.0 M HCl for 16 h at 110 °C and without hydrolysis. Samples were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC Waters AccQTag reagent, PN WAT052880) or with propyl chloroformate (EzFaast™, LC/MS Physiological (Free) Amino Acid Kit KH0-7337) following standardized, validated protocols (Banack and Cox, 2003a; Esterhuizen and Downing, 2008). Standards and blanks were derivatized and analyzed separately and in combinations to evaluate chromatographic separation.

2.3. Ultra high pressure liquid chromatography-Tandem mass spectrometry (UHPLC-MS/MS)

AQC derivatives were analyzed using a triple quadrupole instrument (Thermo Scientific Finnigan TSQ Quantum UltraAM, San Jose, CA) after separation by a Waters Acquity-UHPLC system with a Binary Solvent Manager, Sample Manager and a Waters AccQTag Ultra column (part# 186003837, 2.1 × 100 mm) at 55 °C. Separation was achieved using gradient elution at 0.65 ml/min in aqueous 0.1% (v/v) formic acid (Eluent A) and 0.1% (v/v) formic acid in acetonitrile (Eluent B): 0.0 min = 99.1% A; 0.5 min = 99.1% A curve 6; 2 min = 95% A curve 6; 3 min = 95% A curve 6; 5.5 min = 90% A curve 8; 6 min = 15% A curve 6; 6.5 min = 15% A curve 6; 6.6 min = 99.1% A curve 6; 8 min = 99.1% A curve 6. Nitrogen gas was supplied to the heated electrospray ionization (H-ESI) probe with a nebulizing pressure of 40 psi and a vaporizer temperature of 400 °C. The mass spectrometer was operated under the following conditions: the capillary temperature was set at 270 °C, capillary offset of 35, tube lens offset of 110, auxiliary gas pressure of 35, spray voltage 3500 V, source collision energy of 0 eV, and multiplier voltage of −1719 V. A divert valve was used except during the selected ion monitoring (SIM) scans. The second quadrupole was pressurized to 1.0 m Torr with argon. Ion *m/z* 459 was isolated in the first quadrupole filter as the precursor ion and subjected to collision induced dissociation (CID). Second step mass filtering was performed using selective reaction monitoring (SRM) of BMAA after CID in the collision cell. The following *m/z* transitions were monitored: 459–119, CE 21 eV; 459–289 CE 17 eV; 459–171 CE 38 eV. The resultant three product ions originating from derivatized BMAA (*m/z* 119, 289, 171) were detected, after entering the third quadrupole and their relative abundances were quantified. Compound specific *m/z* transitions were added to our standard method, 459–258 CE 21 eV (BMAA), 459–188 CE 38 eV (DAB) and 459–214 CE 35 eV (AEG) to increase selectivity. A new SRM method was established to detect

2,6-diaminopimelic acid using *m/z* 531 as the precursor ion and its most abundant product ions for *m/z* transitions 531–191 CE 21 eV, 531–171 CE 38 eV, 531–361 CE 21 eV, 531–387 CE 21 eV. In order to detect AQC-derivatized compounds of different molecular mass and therefore determine their retention times, the instrument was run in SIM mode using *m/z* 303 (Asn), 317 (Gln), 375 (Trp), 459 (BMAA, 2,4-DAB, AEG), 473 (Orn), 487 (Lys), 489 (2,4-diaminosuccinic acid), 531 (2,6-diaminopimelic acid), 581 (Cys) and 609 (hCys). Common product ions for each molecular ion were established by sequential direct infusion of AQC-derivatized compounds using collision energies 17, 21, 38 eV. Common product ions for the isomers BMAA, 2,4-DAB, AEG, as well as 2,6-diaminopimelic acid were also established by sequential direct infusion of underivatized compounds using collision energies 17, 21, 38 eV. Methionine methylsulfonium was analyzed with the precursor ion set at *m/z* 334 using SIM mode and by infusion as an underivatized amino acid.

2.4. Ultra high pressure liquid chromatography-mass spectrometry (UHPLC-MS), propyl chloroformate derivatization

Chloroformate (EzFaast™) derivatized samples were analyzed with a Thermo Scientific Finnigan TSQ Quantum UltraAM after separation by a Waters Acquity-UHPLC system with a Binary Solvent Manager, Sample Manager, and a Phenomenex AAA-MS 250 × 2.0 mm, 4 μm amino acid column (PN: 00G-4402-B0) with a flow rate of 0.25 ml/min, 10 mM ammonium formate in water (Eluent A), 10 mM ammonium formate in methanol, and the following gradient: 0.0 min = 32% A; 0.01 min = 32% A curve 6; 13 min = 17% A curve 6; 13.01 min = 32% A curve 6; 17 min = 32% A. The instrument was run in Q1MS mode with nitrogen gas supplied to the H-ESI probe with a nebulizing pressure of 40 psi and a vaporizer temperature of 199 °C. The mass spectrometer was operated under the following conditions: the capillary temperature was set at 270 °C, capillary offset of 35, aux gas pressure of 50, spray voltage 5000 V, source collision energy of 0 eV, and multiplier voltage of −1302 V. Selective ion monitoring mode was used to monitor *m/z* 243 (Asn), 275 (Gln), 333 (BMAA, 2,4-DAB, AEG), 347 (Orn), 361 (Lys), 497 (2,6-diaminopimelic), and 525 (hCys) ions using timed segments to optimize instrument scans.

2.5. Ultra high pressure liquid chromatography with ultraviolet detection (UHPLC-UV)

Chromatographic separation by UHPLC-UV with eluents (A and B) purchased from Waters (composition proprietary) were used according to manufacturers specification. Separation was achieved on a Waters Acquity-Ultra High Performance system with a Binary Solvent Manager, Sample Manager, and Sample Organizer by reverse phase over 9.5 min on a Waters AccQTag Ultra column (part# 186003837, 2.1 × 100 mm) at 55 °C (Cox et al., 2009). The gradient was run with a flow rate of 0.7 ml/min (0.0 min = 0.1% B; 0.54 min = 0.1% B curve 6; 4.04 min = 6.5% B curve 6; 6.04 min = 9.1% B curve 7; 7.74 min = 21.2% B curve 6; 8.04 min = 59.6% B curve 6; 8.64 min = 59.6% B curve 6; 8.73 min = 0.1% B curve 6; 9.5 min = 0.1% B curve 6). The Waters Acquity-Ultra High Performance TUV detector was set at 260 nm.

2.6. Ultra high pressure liquid chromatography with mass spectrometry detection (UHPLC-MS), AQC derivatization

Samples were analyzed with the above UHPLC front-end with a post-column split of the flow delivering a flow rate of 0.3 ml/min to a Waters EMD 1000 single quadrupole mass spectrometer. Nitrogen gas was purified to 97.8% using a NitroFlow nitrogen generator (Parker Balston, Haverfill, MA) and supplied to the electrospray ionization interface (ESI) at 101 psi with desolvation at 500 l/h. Positive ion mode was used to detect derivatized samples using selective ion monitoring at the following *m/z* 303 (Asn), 304 (Asp), 317 (Gln), 318 (Glu), 376 (Trp), 459 (BMAA, 2,4-DAB, AEG), 473 (Orn), 487 (Lys), 489 (2,4-diaminosuccinic acid), 531 (2,6-diaminopimelic acid), 581 (Cys), 609 (hCys) with a span of 0.2 Da and a dwell time of 0.5 s. The source temperature and desolvation temperature were 130 °C and 100 °C respectively. Voltages were as follows: capillary 2.9 kV, cone 27 V, extractor 2 V, RF lens 0.7 V.

2.7. High-performance liquid chromatography with fluorescence-detection (HPLC-FD)

BMAA was separated from other amino acids using a validated method (Banack and Cox, 2003a,b, Banack et al., 2007) by reverse-phase elution (Waters Nova-Pak C18 column, 3.9 × 300 mm at 37 °C) on a Waters 1525 binary HPLC pump and a Waters 717 Autosampler. The mobile phase consisted of 140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2 (Eluent A), and 52% (v/v) aqueous acetonitrile (Eluent B) using a flow rate of 1.0 ml/min, and a 10 μl sample injection. The samples were eluted using a 60 min gradient: 0.0 min = 100% A; 2 min = 90% A curve 11; 5 min = 86% A curve 11; 10 min = 86% A curve 6; 18 min = 73% A curve 6; 30 min = 57% A curve 10; 35 min = 40% A curve 6; 37.5 min = 100% B curve 6; 47.5 min = 100% B curve 6; 50 min = 100% A curve 6; 60 min = 100% A curve 6. Detection was achieved using a Waters 2475 Multi λ-Fluorescence Detector with excitation at 250 nm and emission at 395 nm. Selected chromatographic peaks were collected from HPLC-FD and the mass was verified by injection into UHPLC-MS.

3. Results

Thirteen compounds were analyzed using five different methods adapted from Banack et al. (2007), Esterhuizen and Downing (2008), and Spáčil et al. (2010). In addition, underivatized preparations of BMAA, 2,6-diaminopimelic acid, and methionine methylsulfonium were analyzed. Furthermore, each compound was analyzed using AQC derivatization before and after acid hydrolysis on HPLC-FD, UHPLC-UV, UHPLC-MS, and UHPLC-MS/MS. No appreciable difference was seen in retention times in any of the analytical platforms between hydrolyzed and unhydrolyzed amino acids with the exception of asparagine and glutamine. Following hydrolysis these compounds were

found to revert to their corresponding aspartic (*m/z* 304) and glutamic acids (*m/z* 318). Chromatographic peaks for these two compounds on the HPLC shifted from 12.47 min and 13.97 min (asparagine and glutamine, respectively) to 11.31 min and 12.39 min (aspartic acid and glutamic acid, respectively). On the UHPLC-UV, retention times shifted from 2.14 min and 2.59 min (asparagine and glutamine respectively) to 3.08 min and 3.46 min (aspartic acid and glutamic acid respectively).

BMAA is consistently and clearly separated by HPLC and thus distinguishable from other tested diamino acids and amides by distinct retention time. In the case of methods based on tandem mass spectrometry, the selectivity is increased by additionally using the mass-to-charge ratio of precursor and product ions as well as characteristic ratios between selected SRM traces (Spáčil et al., 2010). Chromatographic separation of BMAA as the sole analyte, versus separation of BMAA within a mixture of thirteen compounds did not affect the retention time of BMAA, its product ion yields or their ratios, when UHPLC-MS/MS was used. Such observation was consistent with expectation and indicates that the presence of these other compounds in the mixture does not interfere with the reliable detection of BMAA.

3.1. Tandem mass spectrometry

All of the compounds tested were clearly distinguished from BMAA using standard methods (Banack et al., 2007; Spáčil et al., 2010) based on triple quadrupole tandem mass spectrometry (UHPLC-MS/MS). In these methods parent ions of AQC-derivatized 2,6-diaminopimelic acid, lysine, ornithine, homocystine, glutamine, cystine, 2,4-diaminosuccinic acid, tryptophan, asparagine, and methionine methylsulfonium were all immediately excluded by the first quadrupole during SRM analysis, due to a different mass-to-charge ratio of their ions with only ions at *m/z* 459 subjected to CID at the second quadrupole. Diamino acid isomers of BMAA all provide ions at *m/z* 459 e.g. 2,4-DAB and N-2(amino)ethylglycine (AEG), but they eluted at

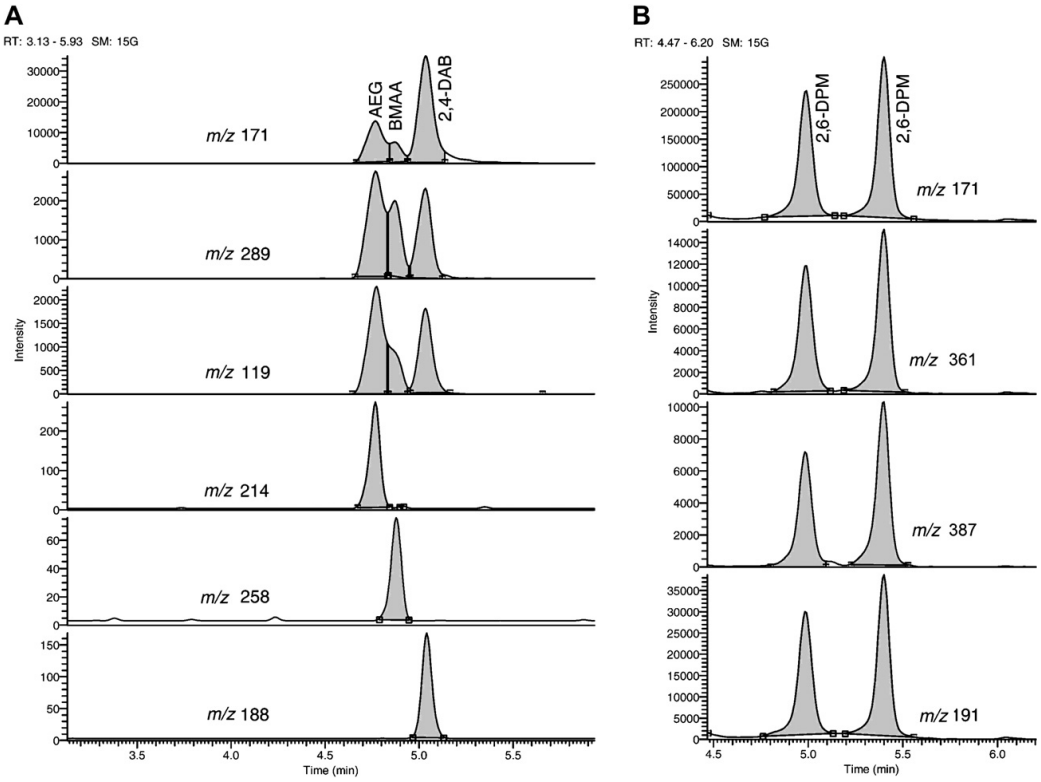

Fig. 1. UHPLC/MS/MS of a non-hydrolyzed 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatized mix of 12 diamino acids. 1A: Given the specific nature of UHPLC/MS/MS using a precursor ion *m/z* 459 and selective reaction monitoring of six transitions *m/z* 459 to *m/z* 171, 289, 119, 214, 258, and 188 respectively only N-2(amino)ethylglycine (AEG), β-*N*-methylamino-L-alanine (BMAA), and 2,4-diaminobutyric acid (2,4-DAB) are visible at retention time 4.77, 4.88, and 5.03 respectively. Distinct product ions and product ion ratios from collision-induced dissociation distinguish these isomers. 1B: UHPLC/MS/MS of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatized 2,6-diaminopimelic acid (DPM) run using a precursor ion of *m/z* 531 and selective reaction monitoring of transitions *m/z* 531 to *m/z* 171, 360, 386, and *m/z* 190 respectively reveal two isomers present in the mix and distinct product ions.

Table 1
Triple quadrupole mass spectrometry of injected and infused non-hydrolyzed AQC-derivatized compounds under mass spectrometric conditions suitable to detect BMAA. Compounds were separated based on retention time, product ions, and product ion ratios. The five most abundant product ions, with areas minimally 1% of most abundant peak, are listed in order of abundance. Product ions *m/z* 171 and *m/z* 145 are fragments of the AQC tag and are not uniquely identifying. Ion ratios represent the relative ratio of the 119 and 289 ion, respectively, in comparison with the most abundant ion 171 (100%).

Diamino acid	*m/z*	Injection	Infusion	Injection	Injection
		RT	Common ions – derivatized	BMAA ion ratio (%) of daughter ion 119 *cf.* 171[a]	BMAA ion ratio (%) of daughter ion 289 *cf.* 171[a]
Asparagine	303	1.68	171, 133, 286		
Glutamine	317	1.99	171, 145, 276, 128, 147		
Methinonine methylsulfonium	334	0.90	129, 128, 198, 266, 143		
Tryptophan	375	6.13	171, 188, 159, 145, 205		
BMAA	459	4.88	171, 289, 119, 145, 258	11.2 ± 0.6	25 ± 1.6
2,4-DAB	459	5.03	171, 376, 315, 145, 336	4.9 ± 0.5	7.4 ± 0.4
N-2(amino)ethylglycine	459	4.77	102, 119, 145, 171, 214	15.2 ± 0.9	19.2 ± 0.5
Ornithine	473	5.77	171, 145, 329, 133, 70		
Lysine	487	6.05	171, 145, 84, 127, 147		
2,4-diaminosuccinic acid	489	3.29	171, 319, 145, 471, 175		
2,6-diaminopimelic acid	531	4.98, 5.38	171, 145, 191, 361		
Cystine	581	6.04	171,411,241, 203		
Homocystine	609	6.08	171, 439, 306, 272, 145		

[a] Mean ± SE.

distinctly different retention times (Fig. 1A). In addition, the ratios from collision-induced dissociation of product ions differ between these amino acids and BMAA (Table 1).

In order to increase specificity, a unique product qualifier ion was selected for BMAA (*m/z* 258) and 2,4-DAB (*m/z* 188) as used by Spáčil et al. (2010). We identified a unique product qualifier for AEG (*m/z* 214; Fig. 1A). Other compounds were analyzed with UHPLC-MS/MS by monitoring additional SRM transitions, which allowed for the detection of these compounds at the different retention times on the LC front-end (Table 1). A new method was established to examine 2,6-diaminopimelic acid using *m/z* 531 as the precursor ion. Two distinct 2,6-diaminopimelic acid chromatographic peaks were observed using AQC derivatization, which correspond to different isomers. Product ions 171 and 145 (Table 1) were fragments of the AQC tag itself and were not uniquely identifying. Derivatized 2,6-diaminopimelic acid provides unique product ions compared to derivatized BMAA (191, 361, *m/z* 387; Fig. 1B).

We characterized product ions of underivatized BMAA and 2,6-diaminopimelic acid by MS/MS product ion analysis using direct infusion of standards into the triple quadrupole working under our standard instrument parameters,

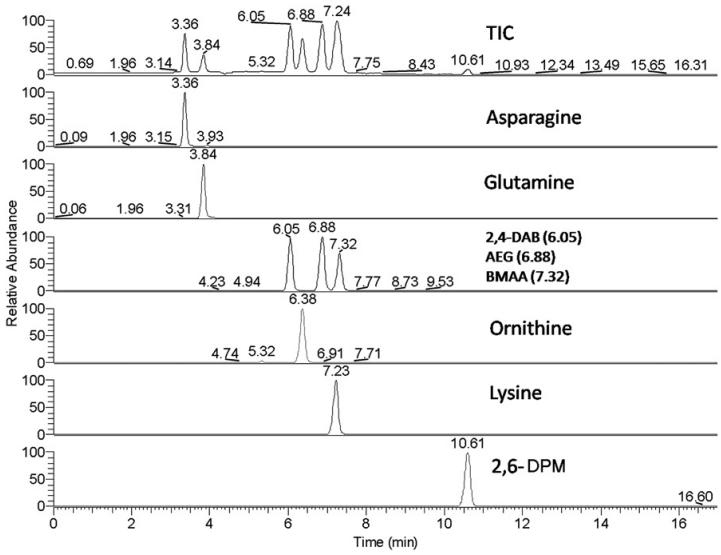

Fig. 2. UHPLC/MS/MS separation of non-hydrolyzed propyl chloroformate (EzFaast) derivatized diamino acids showing the total ion count TIC (top) and the simultaneous selective ion monitoring (SIM) of Asn (*m/z* 247), Gln (*m/z* 275), BMAA, 2,4-DAB, AEG (*m/z* 333), Orn (*m/z* 347), Lys (*m/z* 361), 2,6-DPM (*m/z* 497).

specifically collision energies of 17, 21, and 38 eV, which are used during the identification of derivatized BMAA. The resultant product ions for underivatized BMAA were *m/z* 102, 76, 88, and *m/z* 70 in order of abundance. Similar product ions were used for identification of underivatized BMAA by S. Christensen in Hawaii (*m/z* 102, 88, 76, 73; Bidigare et al., 2009) and by E. Faassen in the Netherlands (*m/z* 102, 88, 76; Faassen et al., 2009). On the other hand, underivatized 2,6-diaminopimelic acid has a different precursor ion and produced completely different product ions (*m/z* 128, 82, 55, and 150) when compared to the product ions used for identification of underivatized BMAA.

We also characterized product ions of AQC-derivatized methionine methylsulphonium, which is distinguished from BMAA in the triple quadrupole UHPLC-MS/MS by mass (*m/z* 334), retention time (0.9 min), and unique product ions from collision-induced dissociation (*m/z* 129, 128, 198, 266, 143, in order of abundance). Underivatized methionine methylsulphonium was infused and was shown to have a parent mass of *m/z* 164 and unique product ions of *m/z* 56, 74, 84, and 28 following collision-induced dissociation. Although not unique, *m/z* 102 is the most abundant ion following CID for underivatized methionine methylsulphonium. Researchers using methods to identify underivatized BMAA should be cautious of this potential overlap.

3.2. Mass spectrometry using propyl chloroformate derivatization

When using propyl chloroformate pre-column derivatization and UHPLC-MS, BMAA clearly separated from the eight compounds tested, based on retention time and mass-to-charge ratio of the precursor ion (Fig. 2). Of the BMAA isomers providing the same ion at *m/z* 333 (2,4-DAB and N-2

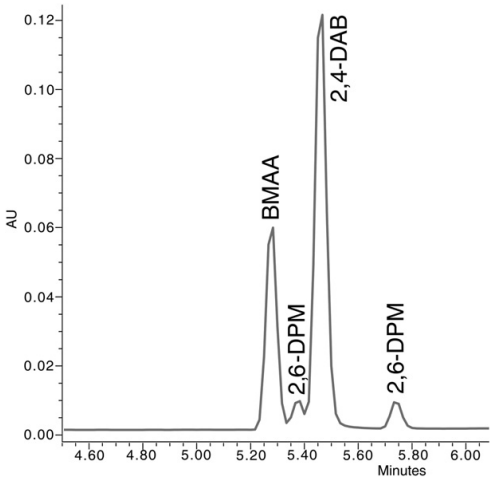

Fig. 3. UHPLC-UV separation of non-hydrolyzed 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatized β-*N*-methylamino-L-alanine (BMAA), 2,6-diaminopimelic acid (DPM) and 2,4-diaminobutyric acid (2,4-DAB).

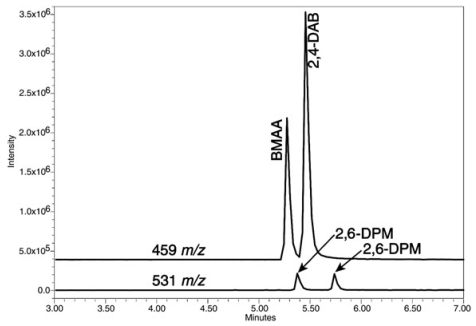

Fig. 4. UHPLC-MS separation of non-hydrolyzed 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatized β-*N*-methylamino-L-alanine (BMAA) and 2,4-diaminobutyric acid (2,4-DAB) (top) detected at *m/z* 459 in selective ion recording (SIR) mode. 2,6-Diaminopimelic acid with a molecular weight of *m/z* 531 can be detected using SIR on a different channel (bottom).

(amino)ethylglycine), differentiation based on retention times was sufficient to distinguish these compounds (Fig. 2) although different product ion ratios do occur as a result of collision-induced dissociation (Banack et al., 2010). BMAA elutes at 7.32 min with AEG eluting 25 s earlier and 2,4-DAB eluting 1.27 min earlier. Furthermore, homocystine elutes at least 5 min after BMAA and requires a modification of the gradient used here to be resolved from other analytes that elute during the re-equilibration phase of the run.

3.3. UHPLC-UV, UHPLC-MS and HPLC-FD separation

In the UHPLC, AQC-derivatized BMAA is separated from 2,4-DAB, 2,6-diaminopimelic acid, lysine, ornithine, homocystine, 2,4-diaminosuccinic acid, asparagine, glutamine, tryptophan, cystine, and methionine methylsulfonium (Figs. 3 and 4; Table 2). Although N-2(amino)ethylglycine is clearly distinguished from BMAA with our current HPLC and

Table 2
Retention times of non-hydrolyzed AQC-derivatized diamino acids and amides showing chromatographic separation in high performance liquid chromatography with fluorescence detection and ultra high pressure liquid chromatography with ultraviolet and mass spectrometric detection. Double chromatographic peaks from 2,6-diaminopimelic acid and 2,4-diaminosuccinic acid were verified by peak collection and mass determination on UHPLC-MS.

Diamino acid	Retention time		
	HPLC-FD	UHPLC-UV	UHPLC-MS
Asparagine	12.47	2.14	2.19
Glutamine	13.97	2.59	2.63
Methylsulfonium methionine	14.89	1.26	1.31
2,6-diaminopimelic acid	19.70, 21.90	5.02, 5.40	5.06, 5.42
2.4-diaminosuccinic acid	22.84, 24.43	4.15	4.18
Cystine	24.27	6.79	6.79
N-2(amino)ethylglycine	29.20	5.04	5.08
BMAA	30.03	5.04	5.08
2,4-DAB	32.24	5.17	5.20
Ornithine	32.80	6.03	6.07
Lysine	33.94	6.80	6.85
Homocystine	34.49	7.77	7.82
Tryptophan	35.73	8.16	8.27

Table 3
An examination of literature citations that examined non-experimental environmental and clinical samples for the presence of BMAA.

Publication	Number of detection methods used	Method description
Vega and Bell, 1967	4	Ionophoresis, Paper Chromatography, Spectrophotometry, ^{1}H NMR
Polsky et al. 1972	1	Amino Acid Analyzer
Dossaji and Bell, 1973	2	Ionophoresis, Amino Acid Analyzer
Kisby et al., 1988	1	HPLC-FD
Duncan et al., 1989	1	Gas Chromatography-MS
Duncan et al., 1990	1	Gas Chromatography-MS
Duncan, 1991	1	Gas Chromatography-MS
Kisby et al., 1992	1	HPLC-FD
Pan et al., 1997	1	Gas Chromatography-MS
Khabazian et al., 2002	1	HPLC-MS/MS
Wilson et al., 2002	1	HPLC-MS/MS
Banack and Cox, 2003a	3	HPLC-FD, Thin Layer Chromatography, Gas Chromatography-MS
Banack and Cox, 2003b	1	HPLC-FD
Cox et al., 2003	2	HPLC-FD, HPLC-MS
Murch et al., 2004a	2	HPLC-FD, HPLC-MS
Murch et al., 2004b	2	HPLC-FD, HPLC-MS
Cox et al., 2005	2	HPLC-FD, HPLC-MS
Banack et al., 2006	2	HPLC-FD, HPLC-MS
Banack et al., 2007	5	HPLC-FD, UHPLC-UV, UHPLC-MS, Amino Acid Analyzer, HPLC-MS/MS
Esterhuizen and Downing, 2008	1	Gas Chromatography-MS
Johnson et al., 2008	4	HPLC-FD, UHPLC-UV, UHPLC-MS, HPLC-MS/MS
Metcalf et al., 2008	2	HPLC-FD, HPLC-MS/MS
Dietrich et al., 2008	1	Gas Chromatography-MS/MS
Rosén and Hellenäs, 2008	1	HPLC-MS/MS
Kubo et al., 2008	1	HPLC-MS
Eriksson et al., 2009	1	HPLC-FD
Scott et al., 2009	1	HPLC-FD
Faassen et al., 2009	1	HPLC-MS
Pablo et al., 2009	2	HPLC-FD, HPLC-MS/MS
Cox et al., 2009	4	HPLC-FD, UHPLC-MS, AAA, HPLC-MS/MS
Craighead et al., 2009	1	UHPLC-MS/MS
Cheng and Banack, 2009	5	HPLC-FD, UHPLC-UV, UHPLC-MS, Amino Acid Analyzer, HPLC-MS/MS
Banack et al., 2009	1	HPLC-FD
Roney et al., 2009	1	HPLC-MS
Bidigare et al., 2009	2	HPLC-FD, HPLC-MS
Caller et al., 2009	1	UHPLC-MS/MS
Moura et al., 2009	1	1H NMR
Krüger et al., 2010	2	HPLC-MS, HPLC-MS/MS
Spáčil et al., 2010	1	HPLC-MS/MS
Brand et al., 2010	2	HPLC-FD, HPLC-MS/MS
Jonasson et al., 2010	1	HPLC-MS/MS
Li et al., 2010	3	HPLC-MS, HPLC-MS/MS, UHPLC-MS/MS

triple quadrupole UHPLC-MS/MS methods, it co-eluted with BMAA in our current UHPLC-UV/MS method. Multiple analytical platforms were used to verify the presence of BMAA in all previous publications using UHPLC-UV or UHPLC-MS (Table 3); based on these criteria, BMAA was present in previously reported samples (Banack et al., 2007; Johnson et al., 2008; Cox et al., 2009; Cheng and Banack, 2009).

High Performance Liquid Chromatography (HPLC) with AQC pre-column derivatization clearly separated the twelve compounds tested from BMAA (Fig. 5, Table 2). Of particular interest, are the BMAA isomers; BMAA eluted at 30 min with AEG eluting 1 min earlier and 2,4-DAB eluting 2 min later. Thus, none of these diamino acids or amides coelute with BMAA using HPLC-FD.

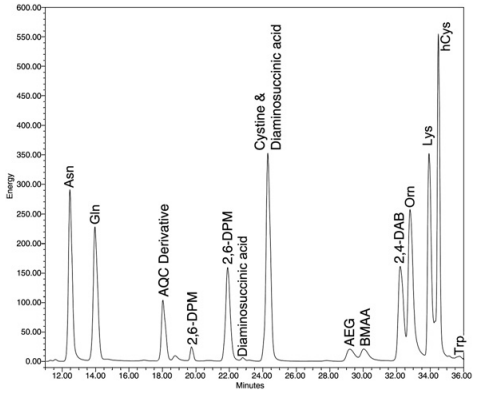

Fig. 5. HPLC-FD separation of non-hydrolyzed 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatized diamino acids: asparagine (Asn), glutamine (Gln), AQC derivative peak (AQC derv), 2,6-diaminopimelic acid (DPM), cystine, 2,4-diaminosuccinic acid, N-2(amino)ethylglycine (AEG), β-*N*-methylamino-L-alanine (BMAA), 2,4-diaminobutyric acid (2,4-DAB), ornithine (Orn), homocystine (hCys), and tryptophan (Trp).

4. Discussion

We found that standard methods of amino acid analysis clearly distinguish BMAA from the twelve amino acids tested. Historically, quantitative analysis of amino acids has been performed by pre- and post-column derivatization to increase selectivity, column retention or chemical stability. Pre-column derivatization has been carried out in order to increase sensitivity and to allow for faster analysis (Cohen, 2001; Liu, 2001). Most commonly, pre-column derivatization of amino acids are performed with chloroformate and AQC (Cohen, 2005; Hušek, 2005; Josefsson, 2005). Similarly, post-column derivatization with ninhydrin has been a mainstay of amino acid analysis (Liu, 2001). In this exercise, we used commercially available AQC and chloroformate derivatization procedures, which allowed us to clearly distinguish BMAA from other diamino acids and amides after HPLC analysis. Furthermore, in the triple quadrupole tandem mass spectrometer, UHPLC-MS/MS, we were able to clearly distinguish underivatized BMAA from underivatized 2,6-diaminopimelic acid which have different product ions resulting from collision-induced dissociation. In all cases, the diamino acids were equally distinguished before and after acid hydrolysis. The triple quadrupole UHPLC-MS/MS is particularly well-suited to detect analytes of low molecular weight within complex matrices (Domon and Aebersold, 2006). In addition, the use of multiple analytical platforms (e.g. HPLC-FD, UHPLC-UV, UHPLC-MS, UHPLC-MS/MS, amino acid analyzer), can result in increased confidence in the determination, verification, and quantification of BMAA, particularly within complex physiological matrices (Table 3; Banack et al., 2007).

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Conflict of interest

None.

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Improved sensitivity using liquid chromatography mass spectrometry (LC-MS) for detection of propyl chloroformate derivatised β-*N*-methylamino-L-alanine (BMAA) in cyanobacteria

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Abstract

β-*N*-methylamino-L-alanine (BMAA) is a difficult molecule to detect, primarily due to its presence in low concentrations in complex matrices. This has resulted in contradictory reports on the presence of BMAA in cyanobacteria. We report improved sensitivity of detection using propyl chloroformate derivatisation, liquid chromatographic (LC) separation, and single quadrupole mass spectrometry (MS) detection. Triple quadrupole mass spectrometry (MS/MS) was used to confirm the identity of BMAA in cyanobacteria based on product ions. We show a 10-fold increase in sensitivity with the LC-MS method compared to the previously published gas chromatography mass spectrometry (GC-MS) method with pre-column derivatised BMAA using a commercially available amino acid derivatisation kit. Clear chromatographic separation of BMAA from 2,4-diaminobutyric acid (DAB), as well as the 20 standard amino acids, was achieved. The analytical method was validated by multiple derivatisation of samples, multiple users, and multiple injections, as well as in various matrices. The quantifier ion used was $[M + H]^+ = 333$ *m/z*. The MS/MS product ions 273 *m/z* and 245 *m/z* were used in identification and peak confirmation. Additionally, we confirm the presence of BMAA in cyanobacteria previously screened with GC-MS as well as the presence of BMAA in newly isolated cultures.

Keywords: β-*N*-methylamino-L-alanine, BMAA, LC-MS, LC-MS/MS

Introduction

The non-proteinogenic amino acid β-*N*-methylamino-L-alanine (BMAA, Fig. 1) is an excitatory neurotoxin, causing neurodegeneration as a result of selective motor neuron death, at concentrations as low as 30 µM (Rao et al., 2006), and damage to motor neurons at concentrations of 10 µM in the presence of other neurotoxic compounds (Lobner et al., 2007). BMAA was first identified in the seeds of *Cycas micronesica*, an indigenous cycad on the island of Guam (Vega and Bell, 1967), where it was initially implicated in the high incidence of amyotrophic lateral sclerosis-Parkinsonism dementia complex (ALS/PDC) (Cox et al., 2005). The link between ALS/PDC and BMAA was based on the presence of free and protein-associated BMAA in the brain tissue of 6 ALS/PDC patients from Guam, but in only 1 of 2 asymptomatic Chamorro brains and not in brain tissues from fatalities of unrelated diseases (Murch et al., 2004a). In support of these data, Pablo et al. (2009) detected BMAA in brain tissue samples of sporadic Alzheimer's disease and ALS but not in Huntington's disease post-mortem samples.

BMAA produced by symbiotic cyanobacteria (*Nostoc*) in *Cycas micronesica* coralloid roots (Cox et al., 2003) was consumed by Chamorro people in the form of flour products made from the cycad seeds. The flour was not considered an adequate source of BMAA as it was washed to remove free cycasin (a cycad glycoside toxin), which also removed free BMAA

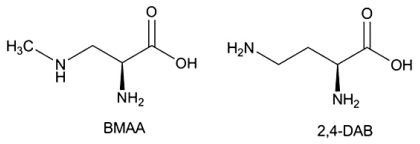

Figure 1
Chemical structures of BMAA and 2,4-diaminobutyric acid (DAB)

(Duncan et al., 1990). However, protein-associated BMAA was not taken into account (Murch et al., 2004b). Additionally, flying foxes (*Pteropus mariannus*), which are also consumed by Chamorro's, forage on the seeds of the cycads resulting in biomagnification of BMAA and exposure of consumers to elevated BMAA concentrations (Cox and Sacks, 2002; Banack et al., 2006). Montine et al. (2005), using an alternative analytical method, reported that no BMAA was detected in the brain tissue of Chamorro ALS/PDC patients. These non-replicated data from a single investigation cast doubt on the BMAA-ALS/PDC link. However, further evidence supporting the link between BMAA and ALS/PDC came in the form of the sudden decline in ALS/PDC occurrence on Guam with a decline in the consumption of flying fox, due to the animal's near extinction (Monson et al., 2003). BMAA was subsequently found in the brain tissue of Canadian Alzheimer's patients (Murch et al., 2004a), suggesting alternate sources of BMAA in the food chain. However, Snyder et al. (2010) reported the lack of BMAA in human brain samples using a 2-dimensional gas chromatography coupled with time-of-flight mass-spectrometry analysis followed by a targeted Parallel Factor Analysis

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deconvolution method. Similarly, several groups have been unable to detect BMAA in complex matrices (Rosén and Hellenäs, 2008; Kushnir and Bergquist, 2009).

Cox et al. (2005) found that culture collection strains of free-living freshwater and marine cyanobacteria from all five taxonomic sections contained BMAA, and concluded that most, if not all, cyanobacteria produce BMAA. Findings by Cox et al. (2005) were confirmed by Banack et al. (2007), Esterhuizen and Downing (2008), Metcalf et al. (2008) and Faassen et al. (2009). Metcalf et al. (2008) and Faassen et al. (2009) reported the presence of BMAA in cyanobacterial surface scum therefore accentuating the risk of human exposure to BMAA. However, the extent of exposure is currently unknown. Recent publications suggest that BMAA is not only present in cyanobacteria, but may also be released into water supplies and reservoirs used by humans (Metcalf et al., 2008; Banack et al., 2007).

There has been considerable controversy over the analytical methods employed for BMAA detection and its role in ALS/PDC. Additionally, the controversy over analysis has resulted in some debate over the validity of the reports on cyanobacteria as the source of BMAA. To date several analytical methods have been published that employ pre-column derivatisation with either ethyl chloroformate or 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) and analysis by UPLC-UV, HPLC-FLD, LC-MS, LC-MS/MS and GC-MS (Banack et al., 2007; Cox et al., 2003, 2005; Esterhuizen and Downing 2008; Spáčil et al., 2010). Post-column derivatisation with ninhidrin and analysis by amino acid analyser (Banack et al., 2007), and methods employing no derivatisation with HILIC LC-MS/MS, have also been reported (Rosén and Hellenäs, 2008; Kubo et al., 2007; Moura et al., 2009; Krüger et al., 2010; Faassen et al., 2009). Only one account where pre-column derivitisation was not used prior to MS detection reported the presence of BMAA in cyanobacteria, but at much lower concentrations than reported with pre-column derivatisation (Faassen et al., 2009).

Recently, Rosén and Hellenäs (2008) reported the co-occurrence of BMAA and DAB in cycad seed and the sole occurrence of DAB in cyanobacterial samples. DAB is a structural analogue of BMAA. Like BMAA, DAB is a neurotoxin, and was first isolated from *Lathyrus latifolius*. Rosén and Hellenäs (2008) suggested that previous reports of BMAA in cyanobacteria might be due to confusion with DAB. Similarly, Krüger et al. (2010) suggested misidentification of DAB and/or tryptophan as BMAA in cyanobacteria. However, Faassen et al. (2009) and Spáčil et al. (2010) reported co-occurrence of BMAA and DAB in various cyanobacterial samples tested.

In order to improve sensitivity, and in effect improve separation of confounder molecules, LC-MS separation with propyl chloroformate pre-column derivatisation was employed to confirm previous reports, screen additional samples, and show adequate distinction between DAB, BMAA and tryptophan in these samples.

Experimental

Chemicals were purchased from Sigma-Aldrich. Stock solutions of BMAA (Sigma-Aldrich, L-BMAA hydrochloride B-107) and DAB (Fluka, L-2-4-diaminobutyric acid hydrochloride #32830) were prepared to a concentration of 100 mM by mixing individual standards in Millipore (16.8 MΩ) water. The EZ:Faast™ LC-MS Free (Physiological) Amino Acids Kit with 250 x 2.0 mm column (Phenomenex KH0-7337) was purchased from Separations, South Africa.

Cyanobacterial cultures representing taxonomic diversity and geographic distribution in Southern Africa were collected from various freshwater impoundments from 2006 to 2007 and made uni-algal by standard methods on BG-11 and BG-11_0 solid media (Rippka, 1988). Cultures were maintained in liquid media at a temperature of 23°C (± 1°C) at a light intensity of 30 to 40 µmol photon m^2 s^{-1} under constant illumination (Triton Dayglo©). Culture purity was confirmed microscopically before BMAA analysis.

The extraction method was adapted from Cox et al. (2003). Cyanobacterial cultures were centrifuged using a Beckmann Avanti J-20 centrifuge at 15 800 G at 4°C for 10 min to collect biomass, which was snap-frozen in liquid nitrogen and lyophilised overnight in a VirTis bench-top freeze dryer (condenser temperature of -51°C and a vacuum of 350 mTorr). BMAA was extracted from lyophilised cultures (20-500 mg dry weight) by sonication (Bandelin sonoplus ultrasonic sonicator, 40% power at 1 x 30 s burst, and a 50% duty cycle pulse) with 0.1 M trichloroacetic acid. Free BMAA was obtained in the supernatant after centrifugation (Beckmann Avanti J-20 at 15 800 G for 3 min at 4°C) to precipitate proteins. TCA (0.1 M) was added to the pellet again to wash off residual free BMAA. The pellet was re-suspended by repeated vortexing and centrifuged as before. Supernatants were pooled and analysed for free BMAA. Protein-associated BMAA was released by liquid acid hydrolysis in 6 M HCl and 2% thioglycolic acid (to prevent tryptophan oxidation) at 110°C for 24 h in an inert atmosphere and filtered through a 0.22 µm filter (Lasec) to remove debris prior to derivatisation.

The EZ:Faast™ amino acid analysis for LC-MS derivatisation incorporates a concentration step on a proprietary sorbent medium to eliminate the effects of interfering compounds, a wash step, elution from, and removal of, the sorbent medium, and sample clean-up by organic extraction as well as derivatisation with a proprietary chloroformate derivative. Figure 2 shows the simplified schematic illustration of how the derivatisation reaction occurs and the BMAA derivative obtained using propyl chloroformate.

BMAA, DAB, tryptophan, and proteinogenic amino acids were analysed using a Shimadzu LC coupled to a Shimadzu MS (2010 EV) after derivatisation using the LC form of the EZ:Faast™ amino acids analysis kit (Phenomenex). BMAA was separated from other amino acids by liquid chromatography on a commercial column (Phenomenex AAA-MS 250 x 2.0 mm

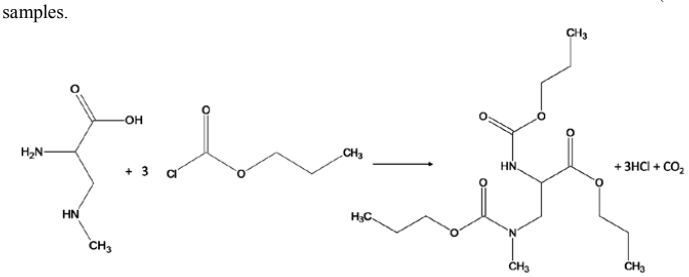

Figure 2
Schematic diagram of the BMAA derivatisation reaction

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amino acid analysis column). A solvent gradient was used with A: 10 mM ammonium formate in water and B: 10 mM ammonium formate in methanol (0.0 min = 68% B, 13.00 min = 83% B, 13.01 min = 68% B, 17.00 min = 68% B) at a flow rate of 0.25 mℓ·min^{-1} and 1 µℓ sample injection volume. Column temperature was kept constant at 35°C. The mass spectrometer electrospray ionisation (ESI) source (positive ion mode) temperature was set at 250°C. The ion scan range was between 20 and 600 *m/z*. The detector voltage was set at 1.5 kV. The interface voltage was set at 4.5 kV and the curved desolvation line voltage at -20 V with the heating block at 200°C. The data was analysed using LC-MS solutions Ver. 3 software.

LC-MS/MS was used to distinguish between DAB, BMAA and tryptophan, which all yield quasi-molecular ions at *m/z* 333 upon propyl chloroformate derivatisation. For LC-MS/MS, samples of free and protein-associated BMAA extracts were derivatised using propyl chloroformate as previously described and separated by HPLC (Water Acquity Ultra Performance LC) on a Phenomenex EZ:Faast AAA-MS column (250 x 2.0 mm) by gradient elution (0.00 min = 68% B, 13.00 min = 83% B, 13.01 min = 68% B, 17.00 min = 68% B) with a mobile phase composition of 10 mM ammonium formate in water (A) and 10 mM ammonium formate in methanol (B) (flow rate: 0.25 mℓ·min^{-1};column temperature: 35°C). Derivatised samples of free and protein-associated cellular amino acids were analysed using a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer with the following settings: ESI voltage set to 5 000, nebulising gas (Nitrogen) at a flow rate of 40, vaporisation temperature of 199°C, capillary temperature at 270°C and capillary and tube lens offsets set to 35 and 70, respectively. Collision-induced dissociation was achieved within the second quadrupole with the precursor ionspecific collision energy set at 12 for the 333 *m/z* precursor ion, and argon gas supplied at 1 mTorr.

The single quadrupole detector (Shimadzu 2010EV) with a Shimadzu LC-20AB liquid chromatography system was validated using a propyl chloroformate pre-column derivatisised BMAA standard (Sigma). The resulting molecule was quantified against 3 internal standards: homoarginine (HARG), methionine-D3 (Met-D3) and homophenylalanine (HPHE). Multiple user, delayed derivatisation and delayed analysis assessments were conducted to verify the system and to determine how robust the method was. BMAA standards were derivatised in varying concentrations on one day and injected in triplicate to assess equipment accuracy and reproducability, and derivatisation of standards in triplicate on 3 consecutive days and by 3 individuals was undertaken in order to determine derivatisation reproducibility. *E. coli* and BSA were used as negative controls for free and protein-associated BMAA, respectively. A matrix of 20 standard amino acids was spiked with the BMAA authenticated standard to test for adequate separation. A calibration curve was constructed for quantification based on the ratio of peak areas of the representative molecular ion for BMAA (*m/z* = 333) to that of Met-D3 (*m/z* = 281). All analyses were conducted in triplicate. Lower limit of detection (LLOD) and lower limit of quantification (LLOQ) values were determined experimentally by dilution to invisibility (S/N < 2 in a matrix) and reproducibility (SD ± 5% in a matrix) respectively.

Accuracy and precision was calculated from the 5 calibration standards of BMAA against the internal standard to validate the method (Table 1). Precision represents a percentage relative standard deviation (%RSD) of the analyte to internal standard peak areas obtained from replicates (*n*=5). Accuracy was calculated as a percentage variation relative to the nominal concentration of each point.

Table 1
Precision and accuracy of calibration standards

Calibration level	BMAA (mg/ℓ)	SD	%RSD	Accuracy (%)
1	0.1489	0.0013	4.7815	87.9
2	0.2977	0.0002	0.6015	94.6
3	0.5955	0.0038	3.6981	98.9
4	1.191	0.0029	1.7383	92.7
5	2.382	0.0045	1.4093	97.9

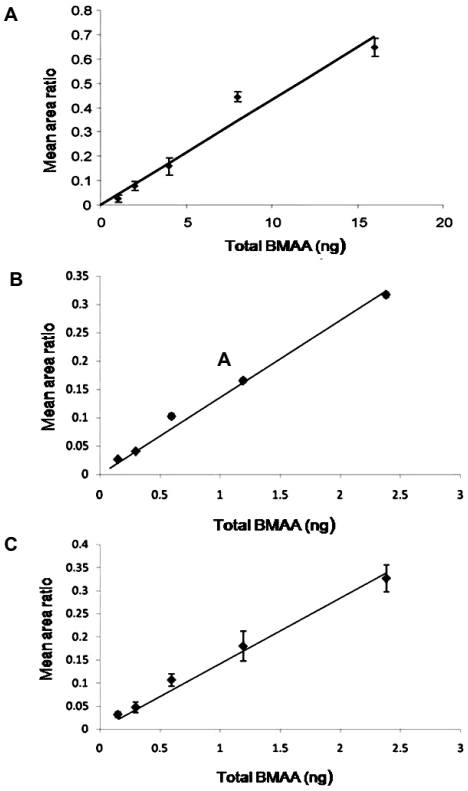

Figure 3
Calibration curve of BMAA standard against the appropriate internal standard. (A) is the standard curve obtained off a Thermofinnigan GC-MS against internal standard of norvaline (NORV), (B) shows the standard curve evaluating the Shimadzu LC-MS machine reproducibility against ISTD methionine-D3 (n=5, r^2=0.989) and (C) shows user validation for derivatisation and analysis on Shimadzu LC-MS (n=5, r^2=0.985).

Results and discussion

Analytical reproducibility was excellent with insignificant variation in individual quantification runs on a given sample (r^2 = 0.989, n = 5). Derivatisation reproducibility across the concentration range used for the calibration curve was acceptable (r^2 = 0.985, n = 5). Figure 3 shows the standard curves obtained using commercial BMAA standard on Thermofinnigan GC-MS (A) and Shimadzu LC-MS for

analysis of intra-day (B) and inter-day (C) variation.

The lower limits of sensitivity of detection and quantification of an authenticated standard for GC-MS analysis were 0.0042 nmol per injection and 0.084 nmol per injection, respectively (Esterhuizen and Downing, 2008). The lower limit of detection in a matrix of 20 µM for each of the 20 standard amino acids was 0.0252 nmol per injection. For the LC-MS method reported here, an LLOD of 0.042 pmol per injection and LLOQ of 0.82 pmol per injection (S/N 2.07) was achieved. The LC-MS method improved the sensitivity 10-fold. LC-MS/MS with single reaction monitoring (SRM) for product ions 273, 245 *m/z* further increased the sensitivity.

Figure 4 shows typical chromatograms obtained for each analytical method. With analysis by GC-MS (A), BMAA had a retention time of 3.95 min and a *m/z* of 130.2. LC-MS analysis (B) yielded a peak at retention time of 9.4 min with a quasi-molecular ion *m/z* of 333 on the Shimadzu LC-MS. Minor variation in retention time in complex matrices was observed. MS/MS analysis yielded a BMAA peak with a RT of 7.37 min, a DAB peak at 6.85 min and tryptophan at 7.95 min.

Despite controversy regarding the validity of BMAA detection methods and possible misidentification of isomers of BMAA, particularly DAB (Krüger et al., 2010) (Fig. 1), the method presented here clearly distinguished between isomers and amino acids with the same quasi-molecular ion (Fig. 5), based on retention time using LC-MS.

Krüger et al. (2010) also suggest that tryptophan, the propyl chloroformate derivative of which shares the same quasi-molecular ion *m/z* with BMAA, could lead to false positive BMAA results. However, as seen in Fig. 5, DAB, BMAA and tryptophan are clearly distinguishable by the RT and, as seen in Fig. 6, clearly distinguishable by the ratio of product ions using MS/MS. Under the conditions used, DAB has a product ion *m/z* ratio (273.1 /245.1) of greater than 2; for BMAA the ratio was

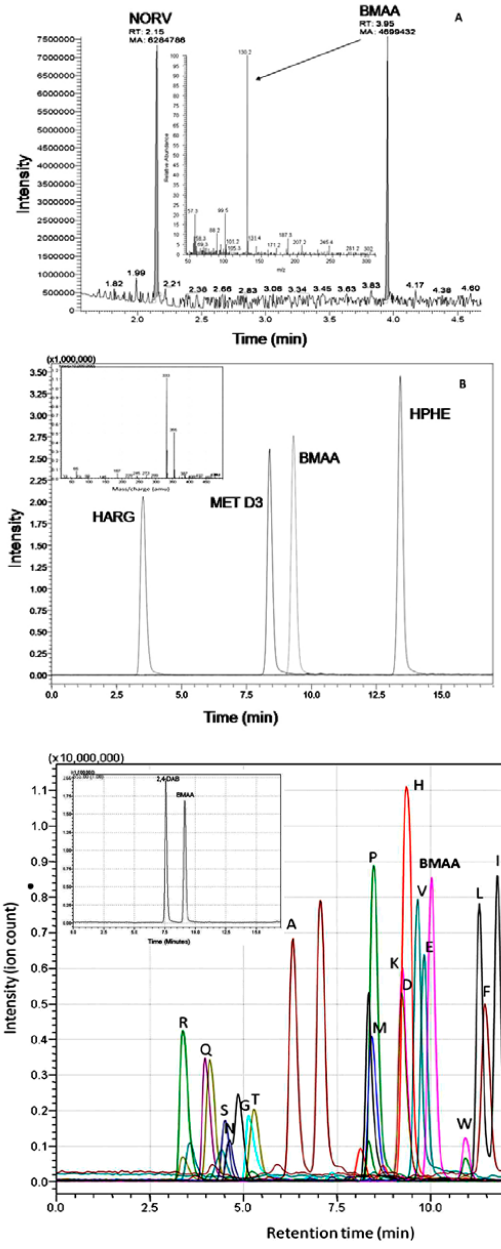

Figure 4 (left)
Typical chromatograms of isolates. (A) Thermofinnigan GC-MS chromatogram (insert is mass spectrum at the retention time of the BMAA standard (Esterhuizen and Downing, 2008). Note the 130 m/z molecular ion. (B) Shimadzu LC-MS chromatogram of the entire run showing only the selected m/z chromatograms of the internal standards and BMAA (insert shows mass spectrum at BMAA elution time). Internal standards are HARG, homoarganine (m/z 317); Met-D3, methionine-D3 (m/z 281); and HPHE homo-phenylalanine (m/z 308).

Figure 5 (left)
SPE recovery of BMAA from an amino acid matrix, detected using a Shimadzu 2010EV LC-MS single quadrupole for propylchloroformate derivatised amino acids. Detection by MS was as follows: Arginine (R) 303 m/z, Glutamine (Q) 275 m/z, Serine (S) 234 m/z, Glycine (G) 204 m/z, Threonine (T) 248 m/z, Alanine (A) 218 m/z, Proline (P) 244 m/z, Methionine (M) 278 m/z, Lysine (K) 361 m/z, Aspartate (D) 304 m/z, Histidine (H) 370, Valine (V) 246 m/z, β-N methylamino-L-alanine (BMAA) 333 m/z, Glutamic acid (E) 318 m/z, Tryptophan (W) 333 m/z, Leucine (L) 260 m/z, Phenylalanine (F) 294 m/z, Isoleucine (I) 260 m/z, Tyrosine (Y) 396 m/z. Cysteine (C) 336 m/z. INSERT: Shimadzu 2010EV MS single quadrupole detection of LC separated propyl chloroformate derivatised BMAA (RT 9.1 min) and DAB (RT 7.6 min) using a 250 x 2.0 mm EZ:faast™ AAA LC column (Phenomenex). Minor ions used for confirmation. m/z 245 used as qualifier ion for BMAA as it is unique to BMAA (m/z 333) and ratio of 273 used for confirmation of DAB (m/z 333).

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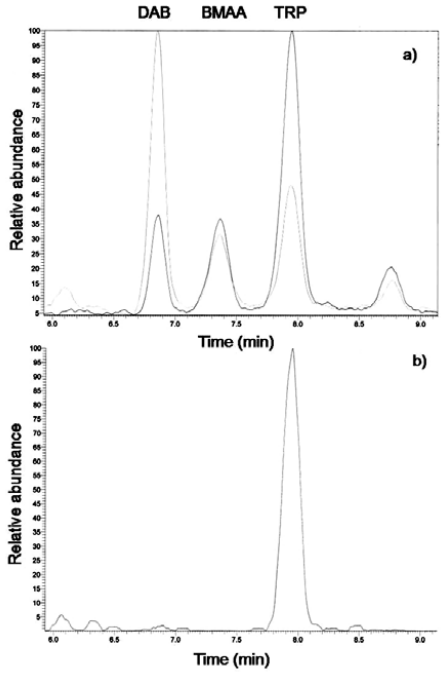

Figure 6
MS/MS chromatograms of product ions (a) m/z 273 (light grey) and m/z 245 (dark grey) , and b) m/z 230, of the precursor ion m/z 333. DAB, BMAA and tryptophan can be clearly distinguished by product ion ratios (DAB has 273>245, BMAA has 273≈245 and tryptophan has 273<245 and a unique product ion of m/z 230.

greater than 0.7 and less than 1.2, whereas for tryptophan the ratio was always less than 0.6.

The derivatisation and detection method described here is available as a standardised kit that is both simple and rapid as well as being extremely sensitive. Final sensitivity is equipment-dependent but detection of BMAA at concentrations below 100 ng ℓ^{-1} should be achievable based on an on-column LLOQ of 0.84 pmol per injection and the solid phase derivatisation, elution and re-suspension system that can concentrate up to 150 times. Additionally, the derivatised samples are stable for several hours. The initial adsorption step takes place on a small quantity of the material, however, possibly limiting use to small volumes or previously concentrated samples. However, the SPE sorbent tip results in additional sample clean-up, together with a wash step and an organic sample extraction. The advantage of the additional purification step is high column efficiency and resolution. This provides complete baseline separation for easy quantitation. Internal standards are used for improved quantitation. The kit facilitates high reproducibility within and between laboratories. Multi-user verification of the method (Fig. 3) yielded a high level of reproducibility.

South African cultures

The presence of BMAA in cyanobacteria, as reported by Esterhuizen and Downing (2008), was confirmed using the propyl chloroformate derivatisation and single quadrupole MS detection after LC separation. Table 2 shows the BMAA content for a range of South African isolates from the cyanobacterial culture collection which had not been previously tested, representing both taxonomic and geographical diversity.

Table 2
The BMAA content of free and protein-associated BMAA in cultures of free-living freshwater South African cyanobacterial isolates not previously tested

Cyanobacterial strain/species	Section (Rippka, 1988)	Origin/ Catchment	Free BMAA (µg/g)	Bound BMAA (µg/g)
Microcystis	I	C811	0.135	NQ
Synechocystis	I	PCC6803	ND	NQ
Synechocystis	I	C522	0.126	ND
Synechocystis	I	M111	0.391	1.290
Oscillatoria	III	C421	NQ	1.227
Oscillatoria	III	C521	0.976	0.936
Oscillatoria	III	C523	ND	1.48
Oscillatoria	III	D231	NQ	0.0518
Oscillatoria	III	D237	0.058	0.179
Oscillatoria	III	D241	0.116	10.616
Oscillatoria	III	G405	ND	0.751
Oscillatoria	III	J121	0.201	ND
Pseudoanabaena	III	A213	NQ	ND
Pseudoanabaena	III	C321	0.876	NQ
Calothrix	IV	D242	0.050	ND
Calothrix	IV	C522	0.060	NQ
Anabaena	IV	A224	0.156	ND
Anabaena	IV	A311	NQ	ND
Anabaena	IV	V202	ND	ND
Anabaena	IV	V203	0.577	1.393

**NQ Detected but not quantifiable*
ND Not detected

Previously, BMAA was detected in 96% (± 2%) of all strains examined (Cox et al., 2005; Esterhuizen and Downing, 2008). With LC-MS detection, BMAA was only detected in 80% (16/20) of freshwater isolates. This difference is, however, statistically insignificant given the limited number of strains tested. Preliminary investigations into the modulation of the BMAA content of cyanobacteria suggest highly variable BMAA concentration as a function of environmental conditions and/or culture age and history (data not shown). This may also explain the reduced percentage of strains with detectable BMAA

The presence of BMAA in a primary producer in aquatic ecosystems raises the question of possible bioaccumulation and biomagnification, as occurs in the Guam ecosystem (Banack et al., 2006, 2007; Banack and Cox, 2003). This possibility increases the risk of human and animal exposure. Rao et al. (2006) and Lobner et al. (2007) showed that very low concentrations of BMAA are required to yield neurological damage and even motor neuron death. The extent of the risk to humans from direct exposure of free BMAA in these waters remains unknown, but may be significant, and the exposure via bioaccumulation and biomagnification may be even greater.

Conclusion

The LC-MS method presented here shows an adequate lower limit of detection and quantification and can be used for the

rapid and reproducible detection and quantification of naturally occurring BMAA, both in its free form within a complex organic matrix or in its protein associated form. The method is relatively simple and has the advantages of being standardised by the supplier and allowing the use of various analytical platforms. Additionally, the method is extremely robust with regard to multiple users and minor variations in use. Whether analysing water or biomass hydrolysate, we recommend dilution to the lower limits of quantification for accurate quantification in a matrix, to avoid matrix effects, particularly competition of SPE binding during derivatisation. Of the South African cyanobacteria tested with LC-MS, 80% contained BMAA. Again, no taxonomic or geographic variation in BMAA content or ratio of free to protein-associated BMAA was observed.

The increasing body of evidence linking BMAA to neurodegenerative diseases, and the potential for exposure to BMAA from cyanobacterial sources, suggests the need for routine monitoring, the development and adoption of safety guidelines for BMAA, and further exposure and epidemiological studies.

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APPENDIX B

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Environmental modulation of microcystin and β-*N*-methylamino-L-alanine as a function of nitrogen availability

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ABSTRACT

The most significant modulators of the cyanotoxins microcystin and β-N-methylamino-L-alanine in laboratory cyanobacterial cultures are the concentration of growth-medium combined nitrogen and nitrogen uptake rate. The lack of field studies that support these observations led us to investigate the cellular content of these cyanotoxins in cyanobacterial bloom material isolated from a freshwater impoundment and to compare these to the combined nitrogen availability. We established that these toxins typically occur in an inverse relationship in nature and that their presence is mainly dependent on the environmental combined nitrogen concentration, with cellular microcystin present at exogenous combined nitrogen concentrations of 29 μM and higher and cellular BMAA correlating negatively with exogenous nitrogen at concentrations below 40 μM. Furthermore, opposing nutrient and light gradients that form in dense cyanobacterial blooms may result in both microcystin and BMAA being present at a single sampling site.

1. Introduction

Cyanobacteria are photosynthetic prokaryotes that produce a range of toxic compounds (Carmichael, 1992), including the heptapeptide, microcystin (MC), and the unusual amino acid, β-N-methylamino-L-alanine (BMAA). MC elicits hepatotoxicity by the inhibition of eukaryotic protein phosphatases (MacKintosh et al., 1990) which subsequently results in the disruption of the liver cytoskeleton (Meng et al., 2011), while BMAA is a potential environmental trigger for the neurodegenerative diseases Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex (ALS/PDC) (Cox and Sacks, 2002), Amyotrophic Lateral Sclerosis (ALS) and Alzheimer's Disease (Murch et al., 2004). The widespread occurrence of toxin-producing cyanobacterial blooms and the associated cost of regular monitoring for toxins has lead to extensive research on environmental factors that modulate toxin production in order to develop predictive models for toxin content (Downing, 2007; Recknagel et al., 2008; Van Ginkel, 2011; Tao et al., 2012). Many parameters have been found to influence MC production in laboratory culture whereas very little is known regarding the parameters affecting the biosynthesis of BMAA in cyanobacteria. More importantly, no dominant environmental modulator of the production of these toxins has been identified in a natural bloom event.

In laboratory culture, nitrogen availability appears to be the dominant determinant of both MC (Downing et al., 2005a, 2005b) and BMAA (Downing et al., 2011) content. Temperature (Watanabe and Oishi, 1985; Van der Westhuizen and Eloff, 1985), light intensity (Van der Westhuizen and Eloff, 1985; Lee et al., 2000; Long et al.,

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2001), growth phase (Lee et al., 2000), growth rate (Van der Westhuizen and Eloff, 1985; Orr and Jones, 1998; Lee et al., 2000; Oh et al., 2000; Long et al., 2001; Downing et al., 2005a), uptake rates of nitrogen and phosphorous (Van der Westhuizen and Eloff, 1985; Orr and Jones, 1998; Lee et al., 2000; Oh et al., 2000; Long et al., 2001; Downing et al., 2005a) and the rates of carbon fixation (Downing et al., 2005b) have all been shown to modulate MC production to a greater or lesser extent. However, Downing et al. (2005b) found that MC production is only enhanced if the nitrogen uptake rate exceeds the specific growth rate and hence results in nitrogen excess in the cell, and in particular, when the cellular N:C ratio exceeds the Redfield ratio as a result of the excess nitrogen uptake or reduced carbon fixation. These data suggest nitrogen as the most significant modulator of cellular MC content in laboratory culture.

In the only published study on nutrient modulation of BMAA, Downing et al. (2011) showed that BMAA is not produced concurrently with other proteinogenic amino acids but is produced upon nitrogen deprivation. Furthermore, cellular BMAA content rapidly decreased upon the addition of ammonia to nitrogen depleted cyanobacterial cultures (Downing et al., 2011). Interestingly, in one study (Phelan and Downing, 2011) the addition of ammonia to the growth medium also resulted in a substantial decrease in MC production under low light conditions.

None of the nutrient modulation has been demonstrated in cyanobacterial blooms. Furthermore, despite the apparently opposing effects of nitrogen on MC and BMAA in separate laboratory experiments, the co-occurrence of BMAA and microcystin has been observed in cyanobacterial blooms, scums and mats from seven inland freshwater impoundments either used recreationally or as sources of drinking water (Metcalf et al., 2008). We therefore investigated MC and BMAA content of crude cyanobacterial bloom material as a function of nitrogen availability at various sites throughout the Hartbeespoort Dam reservoir, South Africa, a freshwater impoundment dominated by *Microcystis aeruginosa* (Zohary and Robarts, 1989; Ballot et al., 2013).

2. Materials and methods

Integrated grab samples of the surface 30 cm (6 L at each site) were taken from ten sites in the Hartbeespoort Dam reservoir during April 2013. Phytoplankton was separated from zooplankton using a light trap as per Larsson et al. (1986). Phytoplankton and zooplankton fractions were frozen, transported at −20 °C, lyophilized and stored at −80 °C until extraction and analysis.

2.1. Extraction and analysis of cyanotoxins

Free and protein-associated BMAA fractions were extracted from lyophilised phytoplankton samples as described by Esterhuizen and Downing (2008) with minor modifications. Free BMAA was extracted by incubation in 0.1 M trichloroacetic acid (TCA) for 30 min, sonication in a waterbath for 10 min, followed by centrifugation at 4000 g for 10 min. Protein-associated BMAA was extracted from resulting cell pellets *via* hydrolysis in 6N HCl at 110 °C for 18 h. Protein hydrolysates were filtered using Ultrafree-MC® 0.22 μM centrifugal filtration units and fitrates were lyophilised and the residues resuspended in 20 mM HCl (Downing et al., 2012) prior to pre-analysis derivatisation using the Phenomenex EZ:faast amino acid analysis kit as per Downing et al. (2011). Free and protein-associated amino acid extracts were analysed using liquid chromatography/mass spectroscopy (LC/MS) as described in Downing et al. (2014). A Phenomenex EZ:faast AAA-MS 250 × 2.00 mm amino acid analysis column (Phenomenex, USA) was used to separate derivatized samples by gradient elution (0.00 min 68%, 13.00 min 83% B, 13.01 min 68% B, 17.00 min 68% B) with the mobile phase composed of (A) 10 mM ammonium formate in water and (B) 10 mM ammonium formate in methanol, at a flow rate of 0.25 mL min^{-1}. BMAA was quantified using electrospray positive ion mode single quadruple mass spectroscopy (Alliance 2695 UHPLC, combined with a Micromass Quattro micro™ Waters).

MC was extracted from phytoplankton in 70% methanol as described by Phelan and Downing (2007) and quantified, in triplicate, using an Abraxis™ Microcystin/Nodularin ELISA assay as per manufacturers' specifications.

2.2. Quantification of environmental nitrogen

Environmental combined nitrate and nitrite concentrations were determined spectrophotometrically using Griess reagent after reduction with copper-coated cadmium using the method described in Benschneider and Robinson (1952). Ammonia concentrations were measured spectrophotometrically using Nessler's reagent as per Leonard (1963).

2.3. Extraction and analysis of chlorophyll a

Chlorophyll *a* was extracted from phytoplankton samples by incubation in 90% methanol at 4 °C for 18 h, and analysed spectrophotometrically at wavelengths 665 nm and 750 nm, using the method and formula for determination of chlorophyll *a* and phaeophytin described by Carvalho and Kirika (2005).

2.4. Statistical analysis

Simple linear regression analysis was conducted using StatsDirect statistical software 2.8.0.

3. Results and discussion

In phytoplankton samples which consisted almost exclusively of *Microcystis aeruginosa*, the maximum detected cyanotoxin concentrations were 252 ng g^{-1} dry weight BMAA, and 24.4 ng g^{-1} dry weight MC. BMAA was detected at 70% of the sites analysed and MC at 50% of the sites. The apparent strong correlation of nitrate plus nitrite with BMAA (Fig. 1(A)) compared to that of ammonia (Fig. 1(B)) can be attributed to the relatively low levels of ammonia compared to nitrate. The strongest correlation with BMAA was however with total combined-nitrogen (r^2 = 0.986,

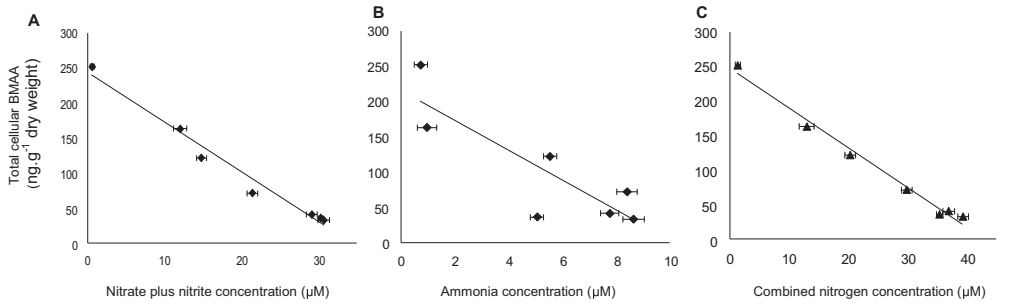

Fig. 1. The correlation between total cellular BMAA in phytoplankton and the availability of exogenous combined nitrogen in terms of (A) nitrate plus nitrite ($r^2 = 0.976$, $p < 0.001$, $n = 3$); (B) ammonia ($r^2 = 0.734$, $p < 0.02$, $n = 3$) and (C) total combined nitrogen ($r^2 = 0.986$, $p < 0.001$, $n = 3$). Error bars denote standard deviations, where $n = 3$. Individual BMAA concentrations represent a single integrated sample (6 grabs) at one sample site.

$p < 0.001$, $n = 3$). The total cellular BMAA in phytoplankton ranged from 252 ng g^{-1} dry weight to 33.85 ng g^{-1} dry weight and was clearly inversely related to exogenous combined nitrogen availability (Fig. 1(C)). This corresponds to laboratory experiments on nitrogen deprivation-induced BMAA production and the rapid disappearance of BMAA on the addition of combined-nitrogen (nitrite, nitrate and ammonia) deprived cultures (Downing et al., 2011).

MC was observed (Fig. 2) at environmental nitrate concentrations >29 μM, and cellular MC increased as nitrate concentrations increased ($r^2 = 0.9431$; $p < 0.001$, $n = 3$) up to 37.3 μM (Fig. 2). MC was only present in phytoplankton when environmental nitrate concentrations were above a threshold of ~30 μM, clearly depicted in Fig. 2. These data closely resembles data presented by Downing et al. (2005b) where a dramatic increase in MC levels corresponded to a ratio of nitrogen assimilation rate to carbon fixation rate in excess of the Redfield ratio. No significant relationship was found between environmental ammonia and cellular MC content in these environmental samples (data not shown) despite reports of reduced MC in the presence of ammonia (Phelan and Downing, 2011).

A distinct inverse relationship existed between cellular MC and BMAA content, clearly illustrated in Fig. 3. Although

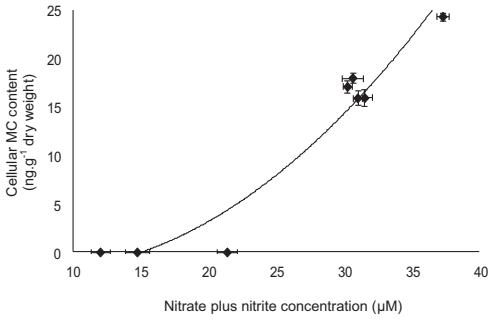

Fig. 2. The correlation between cellular MC content of phytoplankton and environmental nitrate plus nitrite availability (sigmoidal fit: $r^2 = 0.9431$; $p < 0.001$, $n = 3$). Error bars denote standard deviations, where $n = 3$.

both BMAA and MC were present in phytoplankton in integrated samples from two sites, overall, BMAA and MC were not simultaneously present in any other phytoplankton samples analysed. The presence of both cyanotoxins in phytoplankton from two sampling sites (site 4 and 5, Fig. 3), as observed by Metcalf et al. (2008) in cyanobacterial mats and scums, can be attributed to these sampling sites having dense surface bloom layers, which are typically associated with having low nutrient concentrations at the surface of the phytoplankton layer and increasing nutrient concentrations with depth below the surface layer (Klausmeier and Litchman, 2001). The high density of the bloom, the large sampling volume (6 × 1 L at each site) and the depth of sampling, allowed for the simultaneous collection of both nitrogen deprived cyanobacterial cells from the top of the bloom layer and cyanobacterial cells growing in a nitrogen rich environment (>30 μM) below the layer. Thus, due to the large variation of combined nitrogen availability within a bloom at a single site, both BMAA and MC were present within phytoplankton samples, despite a high total combined nitrogen concentration in the integrated samples (>30 μM). For other sample sites, the production of BMAA was modulated by total combined nitrogen availability, and, to a lesser extent, by chlorophyll *a* (Fig. 3). At combined nitrogen levels below 20 μM, BMAA correlated positively to chlorophyll *a*. At high combined nitrogen (>36 μM), BMAA was not detected or was only detected because of the very dense bloom as discussed above. Where high combined nitrogen was found with relatively lower chlorophyll *a* concentrations, no BMAA was detected.

Knowledge of environmental parameters that modulate cyanotoxins allows for the early prediction of toxin presence, as well as precautionary risk management for cyanotoxin exposure.

Predictive methods, such as the use of models for forecasting of ecosystems as described by Recknagel et al. (2008) and Van Ginkel (2011), depend on environmental parameters such as water temperature, phosphorous and nitrate to predict the concentration of both biomass and toxins. Downing (2007) suggested the development of a predictive model for MC, based on growth rate, environmental nitrogen and phosphorous. This study confirms the

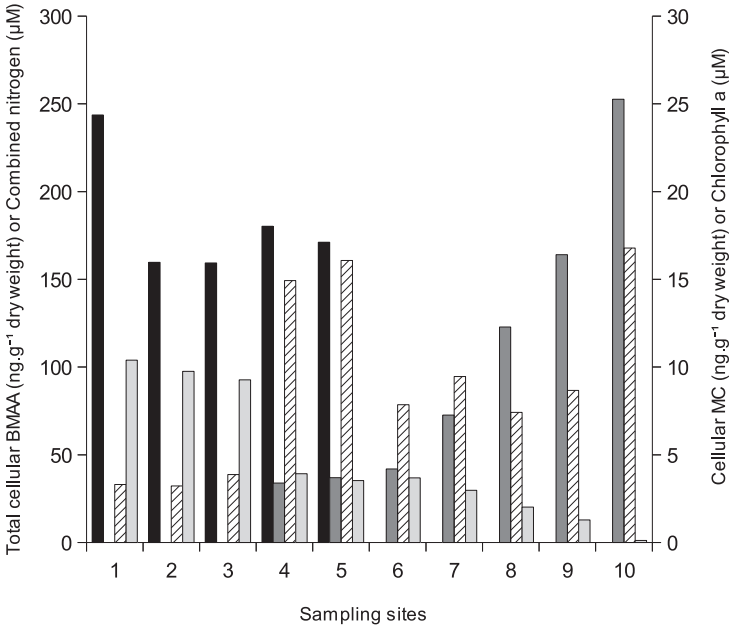

Fig. 3. The relationship between cyanotoxins, MC (black bars) and BMAA (dark grey bars), and the total environmental nitrogen availability (light grey bars) and chlorophyll *a* (diagonally striped bars), at different sampling sites in the Hartbeespoort Dam reservoir, South Africa.

validity of combined nitrogen, chlorophyll *a* and potential for carbon fixation, as valuable parameters in the development of predictive models for both MC and BMAA content of aquatic toxigenic cyanobacterial blooms.

4. Conclusion

We established that in the field, in accordance with laboratory culture studies, nitrogen is the most significant modulator of both MC and BMAA content. Data indicate an inverse relationship between the cellular content of MC and BMAA as a function of environmental combined nitrogen availability. Furthermore, cellular BMAA levels in the presence of moderate combined nitrogen levels (1–30 µM) are almost directly inversely related to the nitrogen concentrations whereas at high nitrogen level (>40 µM) BMAA production is suppressed. This may indicate a direct inhibition of BMAA production by nitrate or ammonia as suggested by Downing et al. (2011) and a positive feedback for BMAA production under nitrogen deprivation. This is the first report of BMAA variation as a function of nitrogen availability in a natural system and the observed trend of nitrogen modulation of BMAA content closely resembles that previously published for laboratory cultures. MC data from this study also supported the observation by Downing et al. (2005b), in which MC is only produced at a specific threshold nitrogen level where the rate of increase of nitrogen in the cell exceeds the carbon fixation rate. In addition, data also showed that, despite having opposing nutrient requirements, the formation of nutrient gradients in dense cyanobacterial blooms may lead to the presence of both MC and BMAA at a single site.

An understanding of the environmental conditions effecting toxin production allows for the development of suitable predictive models for these toxins. However, a more comprehensive study of all the potential modulators of cyanotoxins in the environment, which include phosphorous and carbon fixation, is required for the formulation of accurate predictive models.

Conflict of interest

None.

Transparency document

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2014.05.001.

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