Environmental modulation and metabolism of cyanobacterial β-N-methylamino-L-alanine

Report to the
Water Research Commission

by

TG Downing and S Downing
Department of Biochemistry & Microbiology
Nelson Mandela Metropolitan University

WRC Report No. 2065/1/14
ISBN 978-1-4312-0567-7

July 2014

Obtainable from:
Water Research Commission
Private Bag X03
Gezina, 0031

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EXECUTIVE SUMMARY

Background and motivation for the research

β-*N*-methylamino-L-alanine (BMAA) is a neurotoxic amino acid produced by most, if not all, cyanobacteria (Cox et al., 2005; Esterhuizen & Downing, 2008) and linked to amyotrophic lateral sclersosis/Parkinsonism dementia complex (ALS/PDC) (Cox et al., 2003). Cyanobacteria are ubiquitous and cosmopolitan. They occur in freshwater impoundments in very high densities due primarily to eutrophication and this poses a potential exposure risk (Codd et al., 1999). Evidence exists for various mechanisms of exposure to BMAA from cyanobacterial biomass, including ingestion of contaminated water, aquatic organisms and irrigated vegetables, and via inhalation of aerosolized toxin or toxin-containing cells. However, the reported BMAA content of cyanobacteria has varied substantially from none detected, to mg/g dry weight. Typically BMAA content ranges from a few µg/g dry weight to several hundred µg/g dry weight, but nothing is known about the cause of this reported variation in the BMAA content of cyanobacteria, or about regulation of BMAA content or about biosynthesis of BMAA in cyanobacteria. This lack of understanding of the causes of variation and possible environmental modulators makes management of risk exposure almost impossible.

Project objectives

The purpose of this project was to determine causes of variation in BMAA content in cyanobacteria while confirming the cyanobacterial origin of BMAA and elucidating possible biosynthetic mechanisms of BMAA in cyanobacteria.

The specific project aims were as follows:

- To determine the effect of variation in nutrients on the production of BMAA with an emphasis on the effect of nitrogen on BMAA content
- To investigate possible mechanisms of biosynthesis and metabolism of BMAA in cyanobacteria
- To investigate the possibility of estimating BMAA content based on a more easily measurable metabolite.

Additionally, and due to no clear alternative metabolite being identified that was easier to quantify, an assessment of a commercial BMAA ELISA kit, that became available during the course of this project, was added as an aim.

Summary of the major results and conclusions

BMAA was shown to be a product of cyanobacterial metabolism. This was achieved by inducing production of BMAA using a stable isotope of nitrogen in the form of ammonia as a supplied nutrient, and observing labelled BMAA production as a consequence.

BMAA is environmentally modulated. Transient increases were observed as a result of combined

nitrogen deprivation and increased light intensity or medium phosphorous concentration. However, light and phosphorous responses were probably a function of alteration of cellular nitrogen status, as these variables had no effect under nitrogen-replete conditions. Medium combined nitrogen availability resulted in a rapid removal of free cellular BMAA, with ammonia having an almost immediate effect whereas nitrate had a somewhat delayed effect. These data suggest a role for BMAA in nitrogen stress response in cyanobacteria and offer a basis for environmental studies on BMAA content as a function of physicochemical parameters in surface waters. Furthermore, the immediate role of ammonia in the appearance and disappearance of BMAA offers a starting point for investigating BMAA biosynthesis in cyanobacteria. BMAA is therefore a cyanobacterial metabolite produced under nitrogen deprivation. In order to try and understand the biosynthesis of BMAA, feeding experiments with labelled nitrogen were conducted. Isotopically labelled BMAA was synthesised and this and unlabelled BMAA and other potential metabolites were used in feeding experiments under conditions that would induce either BMAA production or BMAA removal.

BMAA is not produced concurrently with proteinogenic amino acid anabolism. BMAA is produced at the onset of nitrogen limitation but is not produced as a catabolic byproduct of serine protease degradation of cellular components, or directly as a result of cyanophycin or phycobiliprotein catabolism. The transient increase of free cellular BMAA observed in cyanobacteria as a function of decreased cellular nitrogen status suggests a regulatory or metabolic intermediate function for BMAA in cyanobacteria. Published hypothesized biosynthetic mechanisms were shown to be incorrect. BMAA biosynthesis does appear to be enhanced by feeding on amino acids that are transamination products of glutamate, suggesting glutamate as a potential precursor for BMAA. Additionally, BMAA appears to serve as an amino group donor in transamination reactions with either 2-oxoglutarate, oxaloacetate or pyruvate; the resulting BMAA keto acid (2-oxo-3(aminomethyl)propanoic acid) is a substrate for either amination or transamination and/or carbamanation and possibly cyclisation, to form a precursor pool from whence BMAA may rapidly be produced when required by the cells. The rapid transfer of the BMAA alpha-amino group to glutamate suggests that BMAA may serve as an amino donor in the reaction catalysed by one of the 2-oxoglutarate transaminases. The ready reversibility of transaminase reactions suggests a keto acid precursor for BMAA with glutamate as the amino group donor. However, this keto acid has yet to be detected, suggesting that its presence is transient and it is itself a product, either an isomer or otherwise altered precursor. The existence of aminated alpha and beta-carbamate derivatives of the BMAA, and the possibility of aminated forms of these molecules, presents several possible precursor molecule options that become available on nitrogen deprivation, such as the possible decarbamation of a deaminated beta-carbamate of the keto-acid of BMAA with subsequent transamination to yield BMAA.

Results from these experiments therefore suggest that BMAA is rapidly produced from another, as yet unidentified, molecule in the cell, possibly by deamination and decarbamation of aminated BMAA carbamates possibly following de-cyclisation. This is supported by the rapid removal of BMAA on

addition of ammonia to the medium and the existence of both the alpha-aminated and beta-aminated ethyl-carbamates of BMAA. Additionally, BMAA is subject to transamination reactions as determined by transfer of alpha ^{15}N to the amino acid pool. Although the corresponding keto acid was not observed, this molecule may itself be subject to modification within the cell, making detection difficult. The nature of any such modification remains unknown.

BMAA remains a molecule of concern. These data show that BMAA appears to have a primary regulatory or metabolic function that explains its almost universal presence within the cyanobacteria. We suggest that the absence of BMAA in wild strains analyzed is probably due to inappropriate environmental or culture conditions and that the absence in certain culture collection strains can be explained by loss of this function due to many generations under optimal growth conditions. The flux between BMAA and its metabolic precursors and products is rapid and environmentally modulated and this needs to be considered when monitoring programs are established. We have no knowledge of the potential toxicity of the metabolites of BMAA or of whether these metabolites are subject to metabolic conversion to BMAA upon ingestion.

Capacity development

The following students completed their degrees working on this project:

- Ms Karli van Rensburg – BSc (Hons)
- Ms Nastasha Matroos – BSc (Hons)
- Mr Patrick Mwanza – BSc (Hons)
- Ms S Downing had her MSc registration upgraded to a PhD based on the work done on this project. She continues to work on aspects of this project and K5/2065 and will complete her degree in 2014.

Recommendations

Our understanding of the transient nature of BMAA in cyanobacteria indicates a requirement for frequent monitoring of cyanobacteria in drinking water sources where elevated chlorophyll content is detected. In the absence of conclusive data on the possible exposure routes and experimental validation of the ALS/PDC theory, it seems prudent to take measures, such as the placement of warning signs, to prevent any exposure. These data offer the first indication of the possibility of developing alert levels for BMAA based on commonly measured physicochemical parameters. The complex nature of the apparent nitrogen:carbon ratio regulation of BMAA production indicates the necessity for a long-term monitoring program wherein all relevant physicochemical parameters are measured in conjunction with BMAA so as to develop an applicable, environmental model for BMAA risk so as to inform an alert level guideline and better manage exposure risk. The insights gained into the possible regulatory function of BMAA in cyanobacteria require an urgent follow-up study to confirm the function of this molecule and thereby supply a fundamental physiological basis for any

environmental parameter-based alert level framework. We therefore recommend:

a) that research into the role of BMAA as a response regulator be completed so as to support environmentally- based models of BMAA presence, and
b) that a long-term monitoring project be initiated to collect adequate data to support or refute the laboratory findings on physicochemical parameter-based prediction of BMAA levels in cyanobacterial blooms.

The current findings, together with the recommended work, will provide a sound basis for an alert level framework for the analysis of BMAA in recreational and potable water resources.

REFERENCE GROUP

Dr K Murray	Water Research Commission
Dr J Molwantwa	Water Research Commission
Dr S Barnard	North-West University
Ms A Swanepoel	Rand Water
Dr C van Ginkel	Resource Quality Services, Department of Water Affairs
Prof H Patterton	University of the Free State
Prof S Barnard	North-West University
Dr S Jooste	Department of Water Affairs
Prof T Downing	NMMU – Project Leader
Mrs S Downing	Nelson Mandela Metropolitan University (NMMU)

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LIST OF ABBREVIATIONS

Ala	alanine
ALS	amyotrophic lateral sclerosis
ALS/PDC	amyotrophic lateral sclerosis / Parkinsonism dementia complex
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate
Arg	arganine
Asp	aspartate
ATP	adenosine triphosphate
BMAA:	β-*N*-methylamino-L-alanine
CDL	curved desolvation line
Cys	cysteine
DAB	2,4-diamino butyric acid
DW	dry weight
ELISA	enzyme linked immunosorbent assay
ESI	electron spray ionisation
GC/MS	gas chromatography – mass spectrometry
Gln	glutamine
Glu	glutamate
HCl	hydrogen chloride
HPLC	high performance liquid chromatography
LC	liquid chromatography
LC/MS	liquid chromatography – mass spectrometry
LC/MS/MS	liquid chromatography – tandem mass spectrometry
MS	single quadrupole mass spectrometry
MS/MS	triple quadrupole mass spectrometry
NADH	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl-D-aspartate
PCF	propyl chloroformate
Ser	serine
TCA	trichloroacetic acid
Trp	tryptophan
UPLC	ultra performance liquid chromatography
Val	valine

1 INTRODUCTION AND OBJECTIVES

1.1 β-N-methylamino-L-alanine: Environmental and Ecotoxicological Risks

Cyanobacteria produce an array of toxins including hepatotoxins, such as the widely documented and extensively researched microcystin, and neurotoxins including saxitoxins, anatoxins and the newly discovered and not well understood β-*N*-methylamino-L-alanine (BMAA) (Codd et al., 1999; Metcalf and Codd, 2009; Cox et al., 2005). The latter, a non-canonical amino acid, was first isolated from *Cycas circinalis* (Vega and Bell, 1968) and has subsequently been linked to the neurodegenerative disease, amyotrophic lateral sclerosis / Parkinsonism dementia complex (ALS/PDC). The compound elicits neurotoxicity by acting as a glutamate analogue at NMDA and AMPA glutamate receptors, in addition to inducing oxidative stress (Lobner et al., 2007; Weiss and Choi, 1989; Rao et al., 2006). A third potential mechanism of neurotoxicity is the misincorporation of the non-proteinogenic amino acid into proteins, which results in protein misfolding (Caller et al., 2009; Dunlop et al., 2013). It is also hypothesized that protein-associated BMAA may act as a neurotoxin reservoir within the brain from which the toxin is slowly released during protein turnover (Cox et al., 2009) causing prolonged excitotoxicity. This hypothesis is supported by the delayed onset of neurodegeneration in individuals exposed to the toxin over time.

The first link between BMAA and ALS was based on the 100-fold greater incidence rate of ALS, compared to elsewhere in the world, among the Chamorro people of the island Guam (Cox et al., 2003; Steele and McGeer, 2008). BMAA bioaccumulates and biomagnifies within the food chain of the Guam ecosystem, exposing Chamorros to high concentrations of BMAA via the consumption of cycad seed flour and flying foxes (fruit bats) which feed on cycad seeds that acquire BMAA from symbiotic cyanobacteria that live within the coralloid roots of the plant (Cox et al., 2003; Cox et al., 2003).

Renewed concern surrounding BMAA was sparked in light of the findings of Cox et al. (2005), confirmed by Esterhuizen and Downing (2008), that showed BMAA presence in most free-living cyanobacteria tested. The widespread occurrence of cyanobacteria and the frequency of cyanobacterial blooms increases the possibility of human exposure to cyanobacterial toxins and subsequently the risk of acquiring symptoms associated with such toxins. Although the link between BMAA and ALS/PDC is based primarily on the Guam ecosystem and high incidence of ALS/PDC in the Chamorro people of Guam, recent indications are that exposure may occur via several routes including recreational use of bloom-containing waters, aerosol exposure to liquid or dried bloom material or cryptobiotic surface mats (Metcalf et al., 2008), or via aquatic food webs (Jonasson et al., 2010; Brand et al., 2010; Downing et al., 2014). The BMAA content of producing organisms is however highly variable, with the same strain producing vastly different amounts of BMAA on occasion, and to date there are no data to explain why this is. Additionally, the controversy around the cyanobacterial origin of BMAA, fueled by the inability of some researchers to observe BMAA in strains reported as producing the amino acid, has slowed research into the biosynthesis of the molecule.

Identification of a biosynthetic mechanism should facilitate rapid identification of regulatory elements so as to help understand the ecophysiology of this important toxin and contribute to the acceptance of cyanobacteria as the environmental origin of BMAA. Since no published results exist on the effect of environmental nutrient levels and the BMAA content of cyanobacteria, this is the logical starting point from which to attempt to explain the discrepancies in BMAA levels in cyanobacteria and even the inability of certain researchers to observe BMAA in cultures or samples of cyanobacteria.

BMAA is also an important eco-toxin that is rapidly taken up by many species of plants (Esterhuizen et al., 2011) and invertebrates (Downing et al., 2014) and ultimately by vertebrates. It results in oxidative stress in all target organisms and severe deformities in fish models tested (Purdie et al., 2009_a; Purdie et al., 2009_b). The consequences on the environment of high BMAA levels in cyanobacteria remain largely unknown for natural systems and, moreover, the variability of the production rate of BMAA makes such studies premature. Thus, despite the potential human risk of exposure and the potential ecotoxicological effects of BMAA, including potential damage to freshwater fisheries, nothing is known about the biosynthesis of the molecule and the environmental factors that modulate the BMAA content of cyanobacteria. An understanding of conditions that lead to an increased BMAA content of cyanobacteria is fundamental to the efficient management of water resources where cyanobacterial blooms occur and to BMAA exposure risk management.

2 LITERATURE REVIEW

2.1 Environmental Modulation of Cyanobacterial Metabolites

Cyanobacteria have the ability to form storage bodies for all macronutrients. Carbon is typically stored as glycogen, nitrogen is stored as cyanophycin, and phosphorous is stored as large polyphosphate granules. Unbalanced growth in cyanobacteria results in increased storage of relevant macronutrients as a mechanism to retain homeostasis of metabolites. The total C:N:P ratio of biomass may therefore differ from the typical Redfield ratio, without a corresponding long term variation in metabolites. However, in addition to regulatory and primary metabolites which are tightly regulated in terms of their relative cellular concentrations, secondary metabolites are either exported or accumulate intracellularly. Cyanobacterial toxins are the most studied of the accumulated non-storage metabolites.

2.2 Environmental Modulation of Cyanotoxins

The regulation of production of cyanotoxins is relatively poorly understood with the exception of microcystin where the cellular quota appears to be environmentally modulated (Downing et al., 2005a). The metabolic regulation of microcystin production as a function of environmental nitrogen:phosphorous ratios is reflected in the nitrogen:carbon ratio correlation with intracellular microcystin content (Downing et al., 2005b). Increasing nitrogen supply results in changes in both microcystin content and the ratio of toxins containing cyanophycin amino acids (Van de Waal et al.,

2010) due to transient imbalances in cellular amino acid ratios.

2.3 BMAA

BMAA is a non-canonical amino acid containing a secondary amine in the side chain (Figure 2.1a). BMAA is capable of forming two carbamate adducts in the presence of bicarbonate. Although it lacks classical structural characteristics for excitatory amino acid receptor activation (see glutamate in Figure 2.1b), in its carbamate form (Figures 2.1e and 2.1d) it appears to destroy neurons via an excitotoxic mechanism (Allen et al., 1995) as do excess glutamate and N-methyl-D-aspartate (NMDA).

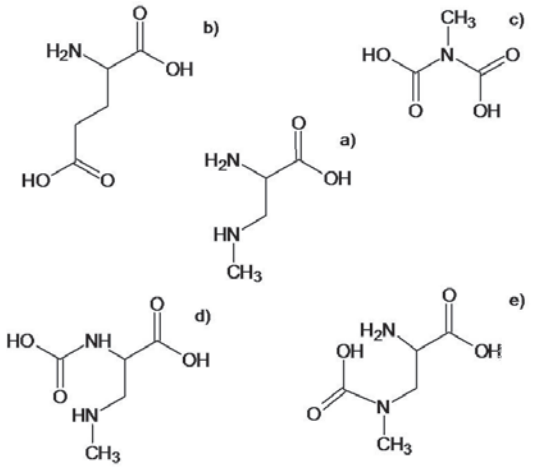

Figure 2.1. Chemical structure of a) β-N-methylamino-L-alanine (BMAA, 2-amino-3-methylamino-propanoic acid or L-α-amino-β-methylaminopropionic acid), b) glutamate, c) N-methyl-D-aspartate (NMDA), d) BMAA α-carbamate and e) β-carbamate.

2.4 Environmental Modulation of Amino Acids

Unbalanced amino acid content can be achieved by nitrogen deprivation followed by resumption of combined nitrogen availability (Van de Waal et al., 2010). The regulation of amino acid metabolism in cyanobacteria appears, however, to be predominantly allosteric (Riccardi et al.,1989) which explains the transience of amino acid imbalance. BMAA may therefore be a product of metabolically regulated efforts of amino acid homeostasis.

2.5 Possible Environmental Modulators of BMAA

The fact that the toxin is very similar structurally to several canonical amino acids strongly suggests an anabolic origin within amino acid metabolism. Brenner et al. (2003) proposed a simple two-step biosynthetic pathway for BMAA in *Cycas* spp., based on expressed sequence tags that rely heavily on the presence of free cellular ammonia for the transamination of any of the proposed starting substrates. It therefore follows that, if the proposed pathway holds true, BMAA production would be up-regulated in the presence of combined nitrogen and therefore positively modulated by the

availability of combined nitrogen. Furthermore, no reliable evidence exists suggesting that BMAA is a cycad product, making the presence of expressed genes in cycads that may possibly be able to catalyse such reactions, irrelevant

2.6 Cyanobacterial Origin of BMAA

Aspects of this work were published in WRC report 1885/1/13 in support of evidence for a large environmental pool of BMAA precursor. We repeat some of the background here because of its relevance to the biosynthesis and environmental modulation of BMAA.

Several independent researchers have detected BMAA in cyanobacteria isolated from marine, terrestrial and/or freshwater environments, using an array of detection methods (Cox et al., 2005; Esterhuizen and Downing, 2008; Metcalf et al., 2008; Bidigare et al., 2009; Faassen et al., 2009; Spáčil et al., 2010, Esterhuizen-Londt et al., 2011). However, some investigators were unable to detect BMAA in either laboratory-cultured cyanobacterial strains and/or cyanobacterial bloom material (Rosén and Hellenäs, 2008; Krüger et al., 2010) resulting in the association between BMAA and cyanobacteria being questioned. The ongoing controversy surrounding the link between BMAA and cyanobacteria relates to possible misidentification of the compound during analysis. This was, however, not the case in those publications cited above (see Banack et al., 2010; Banack et al., 2011). The absence of detection of BMAA when using appropriate methods led to the cyanobacterial origin of BMAA being questioned and although the development of robust analytical methods for the detection of BMAA in a biological matrix has substantiated the link between this neurotoxin and cyanobacteria, evidence of its production from supplied raw materials by cyanobacteria was still lacking. Not only would the demonstration of production of BMAA from raw materials by an axenic culture provide proof of the origin of BMAA, it would also provide valuable insight into the biosynthesis of the molecule.

2.7 Biosynthesis of BMAA

The proposed biosynthetic pathway in cycads (Brenner et al., 2003) is based on expressed gene sequences encoding enzymes that might support a hypothesized pathway. Brenner et al. (2003) proposed a simple 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 this 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.

3 EXPERIMENTAL PROCEDURES

3.1 Cultures 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 maintained in BG11 media (Rippka, 1988) under sterile conditions at a temperature of 23 °C (±1 °C) with constant illumination at a light intensity of 16 mmol m^{-2} s^{-1} (Triton Dayglo®). Culture purity was regularly monitored microscopically and by heterotrophic culture.

3.1.1 Continuous nitrogen excess or deprivation

Cultures of *Microcystis* PCC7806 *Synechocystis* PCC6803, *Anabaena* NMMUCC J331.1 and *Synechocystis* J321 were grown for 12 months under continual nitrogen excess (BG11). The BMAA content of these cultures was monitored periodically.

Cultures grown for prolonged periods under nitrogen excess were collected by centrifugation and re-suspended in either unmodified $BG11_{0,}$ containing no NO_3^+, or modified $BG11_0$ containing no combined nitrogen. Samples were collected periodically and tested for BMAA content.

3.1.2 Short-term nitrogen deprivation of nitrogen-replete cultures

Nutritionally replete mid-log *Microcystis* culture replicates were nitrogen starved for 24 h. Cells were collected via centrifugation (5 000 g for 10 min) and re-suspended to an optical density of 0.6 at 740nm in nitrogen-free $BG11_0$ media (Rippka, 1988). Following nitrogen deprivation, experimental culture replicates were supplemented with 1 mM nitrogen (supplied as either NH_4Cl or $NaNO_3$). Cells were harvested from 10 mL samples by centrifugation (5 000 g for 20 min) at 0 min, 10min, 1h, 4h and 24h.

3.1.3 Nitrogen feeding of nitrogen deprived cultures

In order to analyze the distribution of labelled amino acids, including BMAA, that were released from proteins, or intracellular nitrogen stores, or synthesized 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.

3.1.4 Nitrogen deprivation of diazotrophic cyanobacteria

Anabaena NMMUCC J331.1 was cultured under a range of conditions so as to alter the combined nitrogen availability. Nitrogen-replete conditions included culture in BG11 and in $BG11_0$ where functional heterocysts were allowed to develop once residual ammonia had been assimilated. Nitrogen deprivation was effected by culturing the *Anabaena* in $BG11_0$ under an argon atmosphere, or

in the complete absence of boron so as to reduce the functionality of heterocysts. In order to prevent nitrogen fixation in boron-containing medium, cultures were continually aerated with an artificial gas mixture containing carbon dioxide and oxygen in normal proportions, and argon as a dinitrogen replacement.

3.1.5 Light intensity and quality

Synechocystis NMMUCC J321 was used to evaluate the effects of light intensity and light quality. Cultures were grown under a range of light intensities representing from sub-optimal to saturating conditions. Cultures were also grown under selected photosynthetically active wavelengths; red (600 to 750 nm) and blue (400 to 500 nm).

3.2 Confirmation of Cyanobacterial Origin of BMAA

In order to confirm the production of BMAA by an axenic strain of cyanobacteria from raw materials, an axenic *Microcystis* PCC7806 was cultured in the presence of isotopically labelled ammonium ($^{15}NH_3^+$) as described by Downing et al. (2011).

3.3 Feeding Experiments to Investigate Biosynthesis

3.3.1 Production by primary synthesis with other amino acids

BG_{11} grown mid-log *Microcystis* cultures were nitrogen starved for 24 h prior to exposure to ^{15}N. Cells were collected via centrifugation (5 000 g for 10 min) and re-suspended in combined nitrogen-free $BG_{11}{}^0$ medium. Culture replicates were supplemented with either 1 mM ^{15}N (NH_4Cl) or 1 mM ^{14}N ($NaNO_3$) after nitrogen deprivation, and sampled at 0 min, 10 min, 1 h, 4 h and 24 h.

3.3.2 Production not associated with primary amino acid synthesis

Microcystis PCC7806 cells were harvested after 90 days of growth on ^{15}N containing medium followed and nitrogen deprived as above. Culture samples were taken from the nitrogen free medium at 24, 48 and 192 h. A second experiment was performed using a *Synechocystis* J341 culture, wherein culture replicates were nitrogen deprived for 24 h prior to medium supplementation with either ^{14}N or ^{15}N. Following 10 days of ^{15}N exposure, cultures were nitrogen deprived in $BG_{11}{}^0$ over 120 h.

3.3.3 Feeding experiments

Cultures were exposed to 1 mM of a range of amino acids in order to determine whether any of these, provided in excess, could result in metabolic pressure yielding BMAA. Alanine (Ala), aspartate (Asp), serine (Ser), cysteine (Cys) and tryptophan (Trp) were provided in $BG11_0$ medium, and samples taken as indicated for BMAA and amino acid analysis.

3.4 Sample Preparation

Cyanobacterial cultures were centrifuged using a Beckmann Avanti J-20 centrifuge at 15 800 g at 4 °C for 10 minutes 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 (TCA). 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 analyzed for free BMAA. Protein-associated amino acids were extracted from lyophilised cell pellets using 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 mm centrifugal filtration units and the filtrate dried down using a Savant SpeedVac® Plus after which the dried residue was re-suspended in 200 mL 20 mM HCl.

3.5 Sample Analysis

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 Analysis Kit (Phenomenex KH0-7337) derivatisation procedure 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.

3.5.1 Single quadrupole (MS) analysis

BMAA and other amino acids were separated using a Shimadzu LC20A after partial purification and 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), 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} and column temperature: 35°C). Derivatised samples were analysed using a Shimadzu MS (2010 EV) set to scan in positive mode with the following settings: source voltage 3.5 kV, heating block temperature 200°C, CDL temperature 250°C and detector voltage set to 1.5 kV. BMAA was monitored at 333 *m/z* and mass spectra were checked for product ions and alternative adduct ions such as sodated ions to ensure accuracy of quantitation.

3.5.2 Triple quadrupole (MS/MS) analysis

Samples were derivatised using propyl chloroformate, and separated by UPLC (Waters Acquity Ultra Performance Liquid Chromatography) on a Phenomenex EZ:faast AAA- MS column (250 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} and column temperature: 35°C). Derivatised samples 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 5 000, nebulizing gas (N) 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 parent ion-specific collision energies ranging from 10 to 13, and argon gas supplied at 1 mTorr.

3.6 Production of Molecular Isotopes of BMAA

BMAA isotopes 119.15 amu (BMAA-α^{15}N) and 123.34 amu (BMAA-4,4,4-d_3,$^{15}N_2$) were produced using a proprietary method.

3.6.1 Use of isotopic tracers

Detection and analysis of isotopically labelled amino acids was based on HPLC retention times and mass spectrometric detection of parent ion *m/z* $^{+x}$ amu, where *x* equals 1,2,3,4 or 5. Analysis of the incorporation of $^{+5}$BMAA-originating isotopes into proteinogenic and non-proteinogenic amino acids was based on the relative abundance of $^{+x}$amino acids extracted from $^{+5}$BMAA-exposed cultures, normalised against control cultures. Due to the qualitative nature of this section of the study, the aim being to investigate the metabolism of BMAA in a range of organisms based on the redistribution of $^{+5}$BMAA-originating isotopes, no quantification of labelled or unlabelled amino acids was performed. The data presents the relative abundance of labelled amino acids based on LC/MS peak areas, unless otherwise stated.

3.7 Statistical Analysis

Where appropriate, statistical analysis was performed using Statistica software (StatSoft, Inc. 2002). A one-way analysis of variance (ANOVA) was performed on each dataset to identify if significant differences existed between treatment groups (alpha=0.05).

4 RESULTS AND DISCUSSION

4.1 BMAA is a Cyanobacterial Metabolite

Microcystis PCC7806 exposed to $^{15}NH_4^+$ incorporated ^{15}N into the free amino acids Arg, Gln, Ser, Ala, Asp, Val, and Glu within ten minutes after exposure to the heavy combined nitrogen. The observed increase within 24 hours in ^{15}N in amino acids such as arginine that 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 of the metabolome during the incubation period (Downing et al., 2011). The absence of ^{15}N-labelled BMAA within the incubation period indicates that BMAA, like sarcosine, is not produced concurrently with canonical amino acids.

Nitrogen deprivation of $^{15}NH_4^+$ fed cultures of both *Microcystis* PCC7806 and *Synechocystis* J341 resulted in the appearance of molecular isotopes of BMAA (Downing et al., 2011). This confirmed that axenic cultures of cyanobacteria do produce BMAA from raw materials and that cyanobacteria are a major, if not the primary or sole, source of environmental BMAA. These data not only confirm the cyanobacterial origin of BMAA but also offer insight into possible biosynthetic pathways, environmental modulation, and physiological roles for BMAA in cyanobacteria.

4.2 Environmental Modulation of BMAA

The regulatory mechanisms for nutrient uptake, assimilation and maintenance of stoichiometrically optimal ratios of free and available phosphorous, nitrogen and carbon, are closely connected and are therefore difficult to study in isolation. Despite a tightly controlled homeostasis of primary nutrient uptake and assimilation, a stoichiometric imbalance in availability of certain nutrients may simulate nutrient deprivation and result in a regulatory response to the perceived deprivation (Schwarz and Forchhammer, 2005). Cyanobacteria have the ability to survive long periods of macronutrient deprivation through complex and interlinked regulatory systems that control and modify cellular functions in periods of environmental stress. Nutrient deprivation results in the activation of an array of regulatory cellular responses that, amongst others, limit or arrest cellular growth and significantly change cellular metabolism resulting primarily in the up-regulation of catabolism and a decrease in anabolism. In addition, during macronutrient limitation, cells may alter their photosynthetic apparatus by activating pigment breakdown resulting in a decrease in photosynthetic activity and chlorosis. Nutrient deprivation studies on each of the major physical and chemical parameters were therefore undertaken over different time periods and using strains with different characteristics so as to establish the generality of these observations.

4.2.1 Nitrogen

Amongst cellular responses that are common to all types of macronutrient deprivation, cyanobacterial cells undergo changes that are specific to nitrogen limitation. These include the formation of glycogen granules, in some species the formation of poly-β-hydroxybutyrate inclusions for storage of fixed-CO_2 and in filamentous cyanobacteria, the formation of heterocysts. In cyanobacteria, the primary indicator of the cellular nitrogen status is 2-oxogluterate, which, in turn, is reported to activate the global

nitrogen control system via two response regulators; NtcA and the protein PII. Regulation by this control system occurs predominantly at a transcription level, but also includes some allosteric regulatory aspects. Combined nitrogen, and specifically ammonia, rapidly reduces the BMAA content of cyanobacteria (Downing et al., 2011). This suggests that either BMAA is up-regulated concurrently with other NtcA regulated genes, potentially in a similar fashion, or that BMAA is regulated by an alternative mechanism but in turn regulates the nitrogen regulatory system, or, finally, that the appearance and disappearance of BMAA as a function of combined nitrogen availability is coincidental.

Irrespective of the role of BMAA, results from this study show that nitrogen plays a primary regulatory role in the production of BMAA in cyanobacteria. None of the tested strains grown under fed batch conditions (fed so as to maintain medium combined nitrogen concentrations) produced any quantifiable amounts of free BMAA. However, small amounts of protein-associated BMAA, below the level of quantification, were observed in *Synechocystis* and *Microcystis* strains which could be sampled in sufficient quantities to extract amino acids from more than 500 mg dry weight. In all cultures tested, high extracellular combined nitrogen appeared to prevent the production of free BMAA. Long-term starvation of cultures grown for a prolonged period under nitrogen-replete conditions did result in BMAA production although no significant correlation could be made between the length of feeding and the deprivation time required for production of BMAA. This was presumably due to the ad-hoc nature of maintenance of combined nitrogen concentrations in the long-term fed batch cultures. What could be established was that removal of combined nitrogen from the media did result in an increase in cellular BMAA concentration.

Figure 4.2.1 shows the levels of medium nitrate and ammonia after re-suspension of a washed culture in nitrogen-free medium. The eventual decrease from a maximum nitrate concentration occurred over approximately 450 hours during which time the medium ammonia levels remained fairly constant between 1 and 5 μM, which is limiting. Thus removal and assimilatory reduction of nitrate delayed cellular nitrogen deprivation and corresponding 2-oxoglutarate increases and ammonia decreases until after 150 hours in the nitrogen-free medium.

The link between nitrogen availability and the regulation of photosynthetic activity is fairly well understood in cyanobacteria, with a limitation in cellular ammonia resulting in pigment degradation and chlorosis via the *nbl* pathway. This chlorotic response to nitrogen deprivation in Synechocystis is clearly visible in Figure 4.2.2 where a significant increase in chlorotic cells corresponded with the point of medium nitrate depletion and thus cellular ammonia limitation.

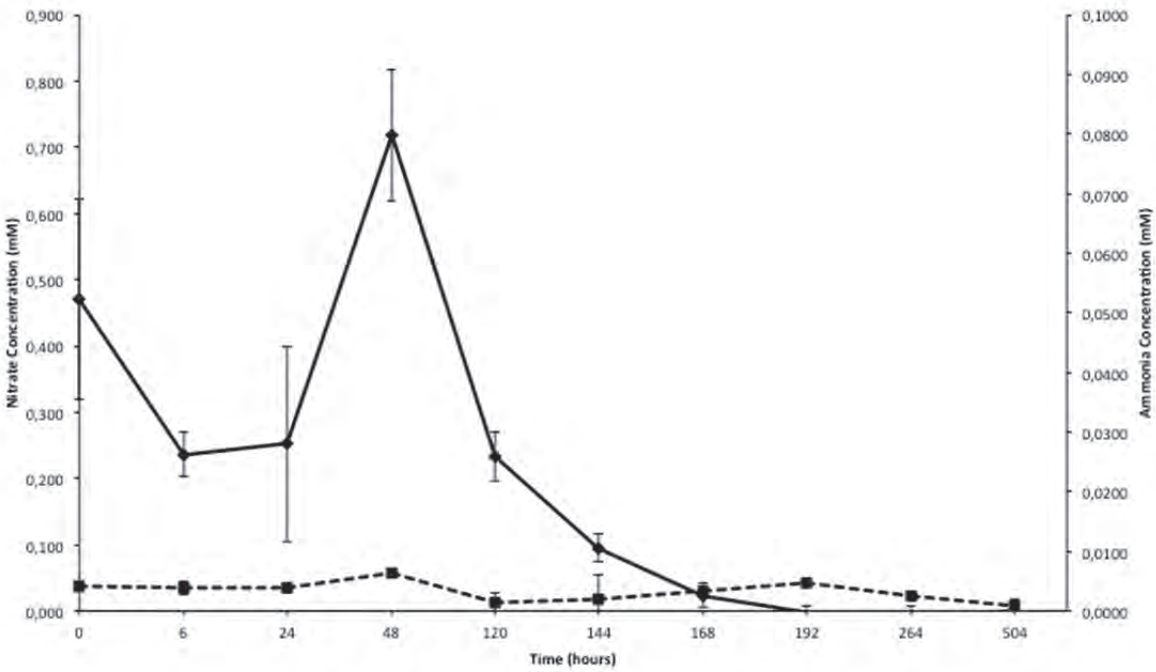

Figure 4.2.1. Combined nitrogen concentrations: nitrate (solid line), ammonia (dotted line), in the growth media of *Synechocystis* J341 over time, following the transfer of nutritionally replete *Synechocystis* cultures into unmodified $BG11_0$.

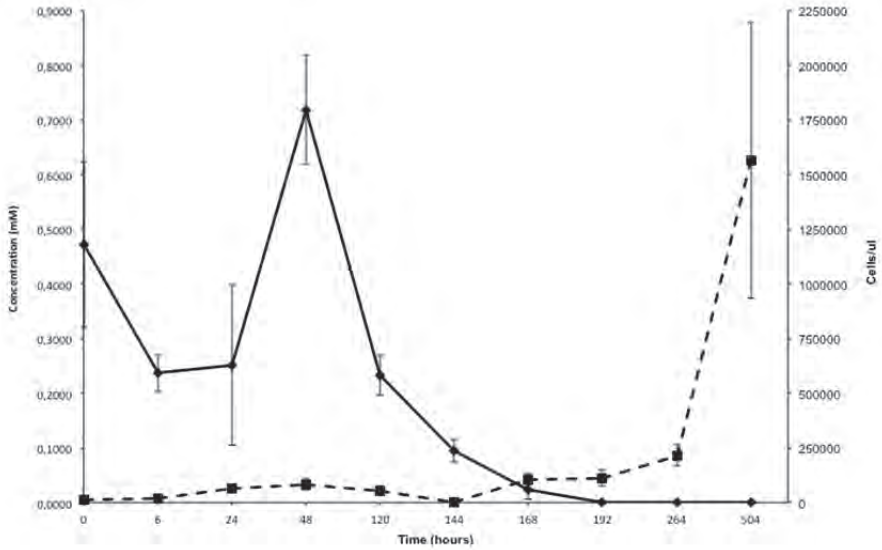

Figure 4.2.2. The correlation between medium nitrate concentration (solid line) and the onset of chlorosis in *Synechocystis* J341 grown in unmodified $BG11_0$ media, where the number of chlorotic cells is indicated as a dotted line.

Figure 4.2.3 shows a steady but small increase in free cellular BMAA as extracellular nitrate levels decreased, with a rapid and significant increase in free BMAA once all extracellular nitrate had been depleted. This increase in free cellular BMAA paralleled an increase in the number of chlorotic cells. The appearance of BMAA at the onset of chlorosis as a function of nitrogen deprivation shows that BMAA is produced specifically when the cell responds to nitrogen deprivation as chlorosis as an active response to this nutritional state.

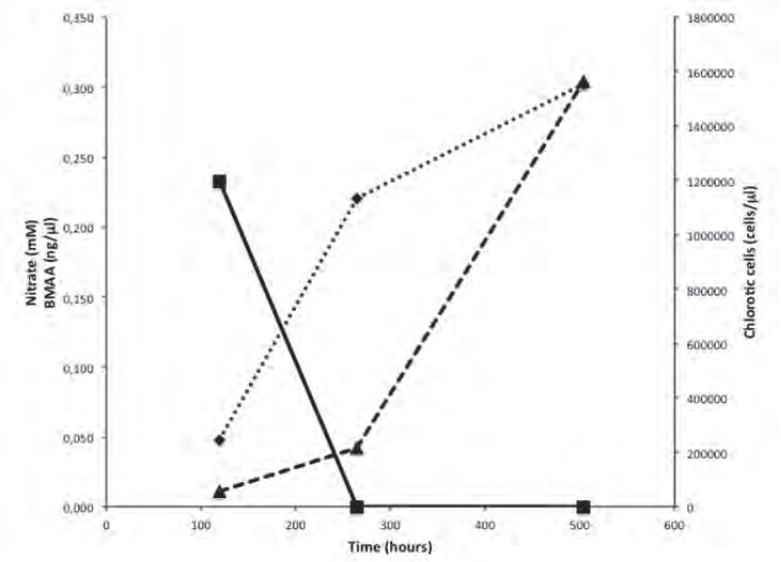

Figure 4.2.3. The relationship between medium nitrate concentrations (solid line) and free cellular BMAA content (dashed line) in *Synechocystis* J341) at the onset of chlorosis (dotted line) when grown in unmodified $BG11_0$.

The correlation between extracellular nitrogen availability and BMAA production was confirmed with a higher temporal resolution. Figure 4.2.4 shows the increase in free cellular BMAA over three days, following the transfer of cells to nitrate-free $BG11_0$ media. Note the initial 24-hour lag period in the production of BMAA.

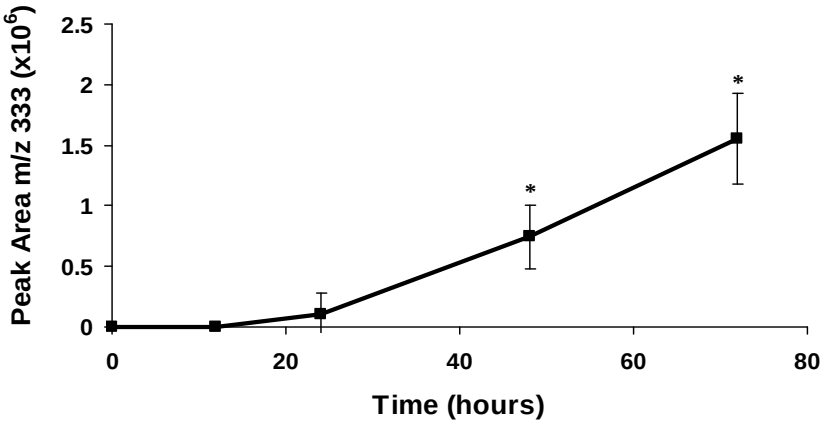

Figure 4.2.4. The increase in free cellular BMAA in *Synechocystis* J341 following 48 hours of nitrogen starvation in cultures grown with NO_3^- as sole nitrogen source. The graph depicts peak areas of LC/MS detection of BMAA parent ion 333 *m/z*, where (*) indicates a significant ($p< 0.05$) increase. Error bars denote the standard deviation of the mean (n=3).

Based on data shown in previous figures regarding the presence of ammonia and residual nitrate in $BG11_0$ media, this lag in BMAA production was attributed to the requirement of BMAA production for the depletion of extracellular nitrogen. Furthermore, the data suggest that it is more specifically cellular ammonia rather than nitrate depletion that results in free cellular BMAA production.

In understanding the role of nitrogen in cellular BMAA quota, the effect of addition of extracellular nitrogen on free cellular BMAA was investigated. The addition of extracellular nitrogen to a nitrogen deplete *Microcystis* culture containing free cellular BMAA resulted in the rapid disappearance of BMAA, with a significantly faster disappearance seen in the presence of ammonia compared to nitrate. This, together with above-mentioned data, confirms that BMAA production in non-diazotrophic cyanobacteria is strongly regulated by the availability of extracellular nitrogen, specifically ammonia.

The rapid disappearance of free cellular BMAA on addition of ammonia did, however, correspond to an increase in protein-associated BMAA (Figure 4.2.6) suggesting that the covalent modification of BMAA that results from ammonia availability results in a form of protein association of the modified molecule. We are unable to provide anything beyond speculation on the biological significance of this observation.

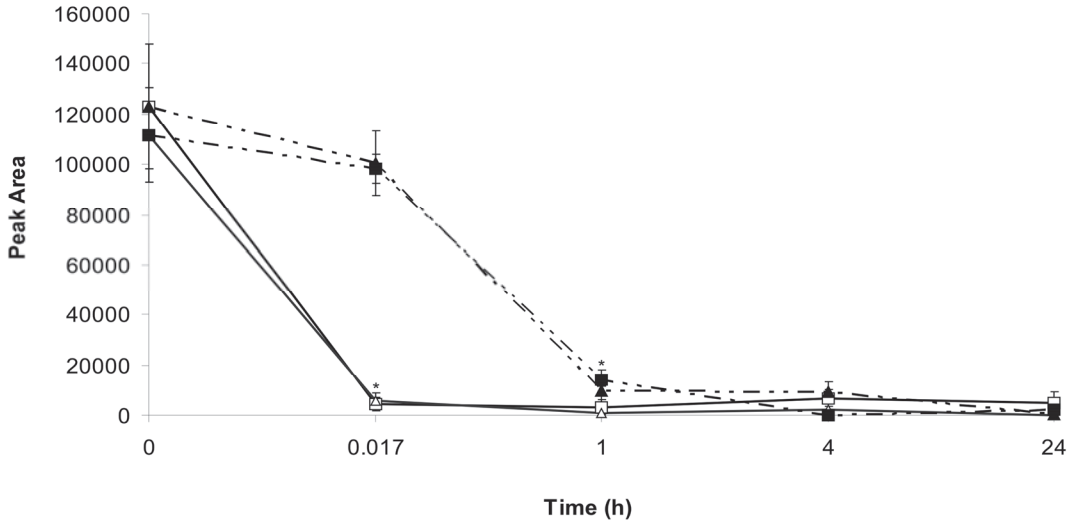

Figure 4.2.5. The disappearance of free cellular BMAA upon exposure of nitrogen starved Microcystis PCC7806 to $15NH_4^+$ (solid line) and $14NO_3$- (broken line), respectively. The graph depicts the peak areas of detection using LC/MS/MS analysis of product ions 245 *m/z* ($14NO_3^-$, solid triangles and $15NH_4^+$, open squares) and 273 *m/z* ($14NO_3^-$, solid squares and $15NH_4^+$, open triangles) produced upon collision-induced dissociation (CID) of the BMAA parent ion 333.1 *m/z*. Error bars denote the standard deviation of the mean (n=3), where (*) indicates a significant ($p< 0.05$) decrease compared to $14NO_3^-$ control samples. (After Downing et al.,2011.)

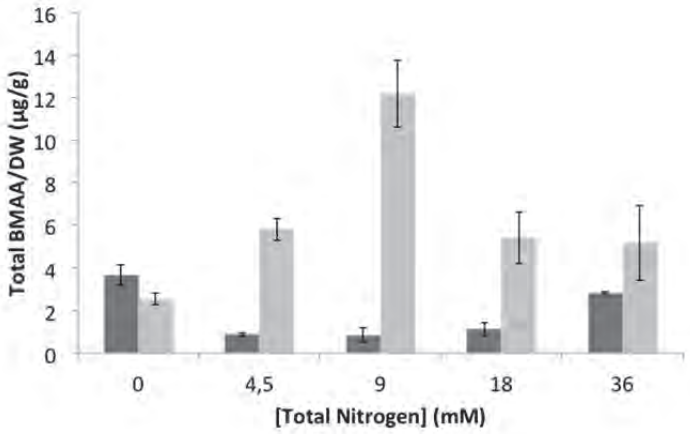

Figure 4.2.6. Free (dark grey) and protein-associated (light grey) BMAA on re-exposure of starved cultures of *Synechocystis* J341 to ammonia for 24 hours. Error bars represent the standard deviation (n=5).

BMAA production in non-diazotrophic cyanobacteria occurred during extreme nitrogen deprivation. The relatively high BMAA content of diazotrophic genera such as *Nostoc* and *Anabaena* (Cox et al., 2005; Esterhuizen & Downing, 2008) raises certain questions regarding environmental nitrogen

modulation of BMAA production since these genera are able to fix nitrogen and presumably do not suffer complete nitrogen starvation except under specific, controlled laboratory conditions. The question therefore arises: does deprivation of extracellular nitrogen result in BMAA production in nitrogen-fixing cyanobacterial strains? In diazotrophic strains, nitrogen is transfered from heterocysts as glutamine (Gln) after amination of glutamate in heterocysts. The vegetative cells therefore typically have a relatively high Glu:Gln ratio, typical of low nitrogen conditions (Flynn and Gallon, 1990). Thus, even though diazotrophic cells are not essentially experiencing nitrogen deprivation, cellular states within the cells may correspond to states characteristic of a cell under nitrogen limitation which in turn could result in relatively high BMAA levels for the actual C:N ratio. The correlation between the Glu:Gln ratio of *Anabaena* NMMUCC J331.1 and the BMAA content is shown in Figure 4.2.7. Higher BMAA values were indeed observed in cultures that had relatively high Glu:Gln ratios with the exception of cultures grown under argon.

These data suggest that it is in fact the relative increase in glutamate as an indicator of nitrogen stress that results in the appearance of BMAA. A direct link between glutamate and BMAA is most easily explained by transamination of 2-oxoglutarate to yield the increased glutamate with a corresponding deamination of a BMAA precursor.

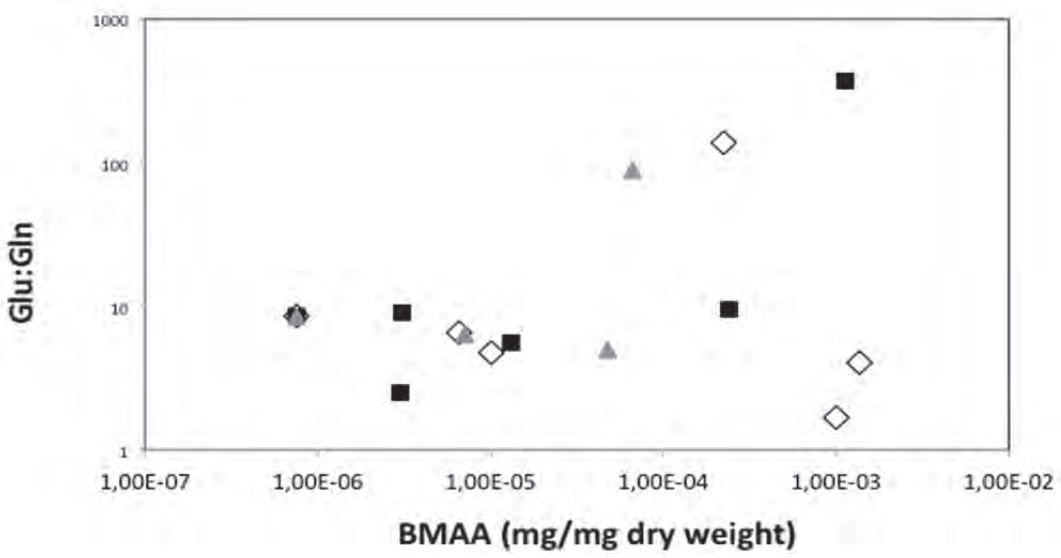

Figure 4.2.7. The correlation between free cellular BMAA concentrations and cellular nitrogen status, depicted as a ratio between free cellular glutamate and free cellular glutamine concentrations, in *Anabaena* NMMUCC J331.1 grown in unmodified $BG11_0$ media (solid square), $BG11_0$ media without boron (grey triangle) and in unmodified $BG11_0$ media in the presence of an artificial gas mixture containing carbon dioxide and oxygen in normal proportions and argon as a dinitrogen replacement (diamond). Glu:Gln ratios are depicted as ratios of peak areas of detection using LC/MS analysis of propyl chloroformate derivatised parent ions 318 *m/z* (Glu) and 275 *m/z* (Gln).

4.2.2 Light

Regulation of cellular pigment composition and photosynthetic activity in response to ambient light

conditions is fairly well documented for cyanobacteria (Grossman et al., 1993; Miller et al., 2002). Photosynthetic pigments function in both photosynthesis and photoprotection, with ambient light intensity being a well-known modulator of pigment metabolism. Regulation of photopigment metabolism implies two main processes: catabolism of photosynthetic pigments, designed to adjust the extent of photosynthetic energy capture and protect the cell from potential photooxidative stress, and anabolism of photoprotective pigments such as carotenoids. The extent of chlorophyll$_a$ and carotenoid metabolism during environmental stresses is highly varied in different cyanobacterial strains (Miller et al., 2002) and additional allosteric modifiers of photosynthetic activity may exist. Pigment composition and concentration changes are not only regulated by light but also other environmental stresses, including, specifically, nitrogen and phosphorous deprivation. Therefore, cellular states that are associated with growth under high light overlap with those characteristics of growth under macronutrient limitations. More importantly, high levels of carbon fixation as a consequence of photosynthesis result in potential unbalanced growth mimicking nitrogen or sulfur stress.

BMAA production in nitrogen-deprived *Synechocystis* J341 cultures increased considerably when culture light intensity was increased, as shown in Figure 4.2.8.

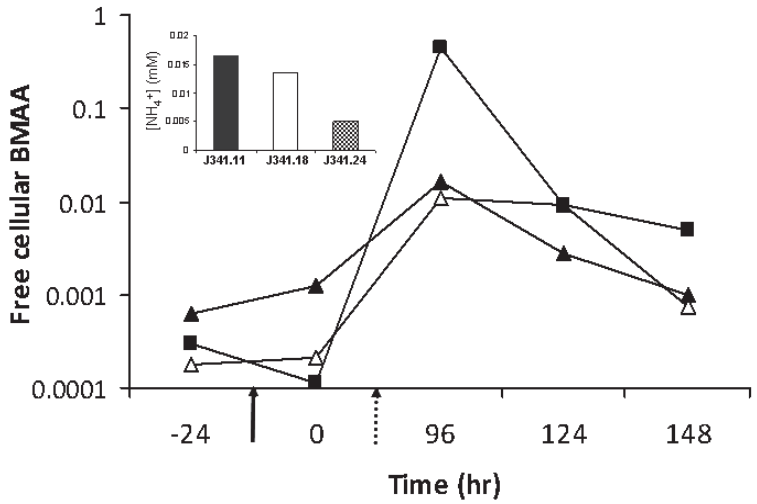

Figure 4.2.8. The flux of free cellular BMAA as a result of nitrogen depletion and light intensity in three different *Synechocystis* J341 cultures over time. Cultures had different nitrogen histories with media nitrogen concentration at T-24h shown as inset. Changes to culture conditions are indicated as follows: BG11 cultures transferred to modified BG11$_0$ (no combined N) (solid arrow); culture light intensity increased (broken-line arrow). Free cellular BMAA is represented as a ratio of product ion 273 *m/z* to internal standard Methionine-D3, using LC/MS/MS analysis as described by Downing et al.(2011).

However, free cellular BMAA levels decreased with continuous growth at a high light intensity. No free cellular BMAA was detected in *Synechocystis* J341 cultures grown under high light in the presence of

combined nitrogen (data not shown). The data suggest a transient status/role for BMAA under high light and nitrogen-limiting conditions with increased light intensity exaggerating the nitrogen deprivation.

The data presented in Figure 4.2.9 further support the transient nature of BMAA increase as a function of increased light. It is particularly interesting to note that red light yielded the lowest BMAA content since red light has been shown to increase the transcription of genes involved in microcystin biosynthesis (Kaebernick et al., 2000) which in turn is primarily metabolically regulated by increased free combined nitrogen and increased cellular N:C ratio (Downing et al., 2005a). It would be particularly interesting to monitor natural populations of microcystin producing cyanobacteria under bloom conditions to evaluate the relationship between the hepatotoxin and BMAA as a function of environmental conditions.

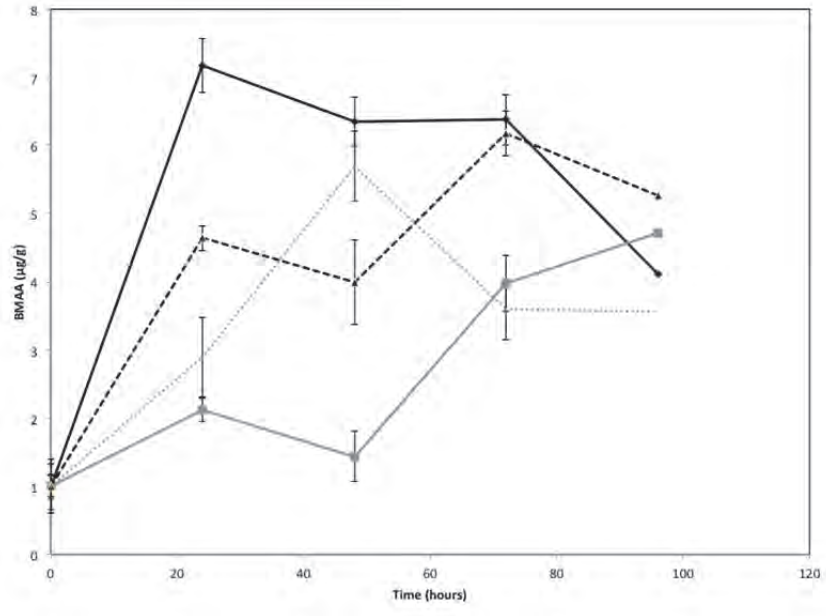

Figure 4.2.9. The effect of light quantity and quality on the BMAA content of nitrogen-deprived cultures of *Synechocystis* J34. The solid black line represents BMAA content on exposure to 18 µmol m^{-2} s^{-1} photosynthetically active radiation encompassing all wavelengths across the spectrum (Triton Dayglo®). The dashed black line represents BMAA content on exposure to 18 µmol m^{-2} s^{-1} blue light (300–500 nm – maximum at 420 nm). The solid grey line represents BMAA content on exposure to 18 µmol m^{-2} s^{-1} red light (600–700 nm). The dotted grey line represents BMAA content in cultures grown in the dark.

4.2.3 The link between light and nitrogen

When considering light and nitrogen modulation of BMAA production, the data show that of these two factors, nitrogen, and specifically ammonia, is the primary environmental modulator of BMAA production in non-diazotrophic cyanobacteria. A high ambient light intensity merely accelerates BMAA production under nitrogen-limiting conditions. From an ecophysiological perspective, light can be considered an indirect modulator of BMAA production under conditions that are typical in planktonic

blooms. High photosynthetic activity in planktonic blooms rapidly depletes available combined nitrogen and alters the cellular carbon:nitrogen balance, which could activate responses characteristic of conditions under which BMAA production occurs.

The apparent partitioning of BMAA between the free and protein-associated forms as a function of continued exposure to a range of nitrogen concentrations (see Figure 4.2.6) may indicate a partitioning driven by nitrogen:light ratio, or a metabolic channeling of BMAA as a function of carbon:nitrogen balance. This is further supported by the results of phosphate modulation where increased environmental phosphate, under non-saturating high light conditions, transiently increased the cellular BMAA content. This was attributed to the positive effect of phosphorous on carbon fixation.

4.2.4 Phosphorous

Polyphosphates, apart from being phosphorous storage molecules, may also serve regulatory functions or act as ATP substitutes (Schwarz and Forchhammer, 2005).

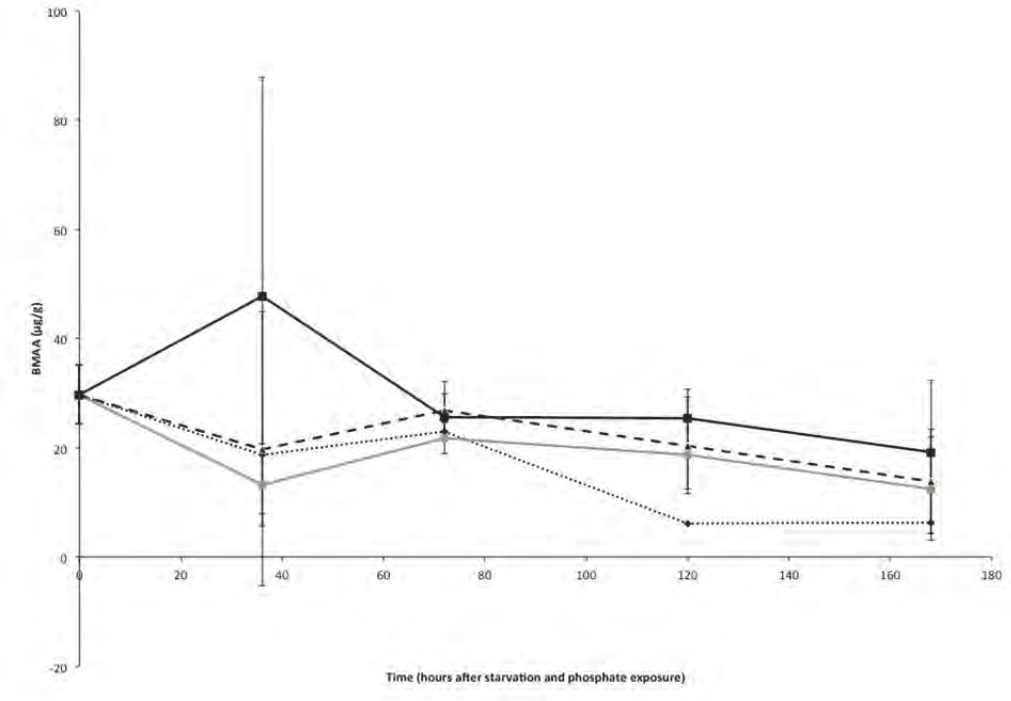

Figure 4.2.10. Total BMAA content of *Synechocystis* grown in combined nitrogen free medium containing either 0 (solid grey line), 0.5 (dotted black line), 1 (dashed black line) or 2 (solid black line) mM phosphate. Error bars represent standard error (n = 5).

Cyanobacterial cells respond differently to various macronutrient limitations. Phosphorous-limited cells tend to contain less chlorophyll$_a$ compared to nitrogen-limited cells and tend to become less chlorotic due to limited phycobilisome degradation and retention of higher phycocyanin levels,

resulting in a higher phycocyanin:chlorophyll$_a$ ratio compared to cells under nitrogen limitation (Collier and Grossman, 1992). In planktonic cyanobacterial blooms, growth of diazotrophic species tends to be phosphorous limited, whereas the growth of non-diazotrophic strains is nitrogen limited.

Increased medium phosphate results in insignificantly increased BMAA content in *Synechocystis* grown in the absence of combined nitrogen (Figure 4.2.10). This corresponds to the light-induced increase in BMAA under nitrogen deprivation and supports the conclusion that cellular nitrogen status, as detected by the cyanobacterial regulatory system (cellular ammonia and the 2-oxoglutarate levels being the primary nitrogen status signals), is the major modulator of BMAA content. Furthermore, the absence of an effect of phosphate in the presence of combined nitrogen (data not shown) confirms that it is nitrogen specifically that is controlling BMAA content.

BMAA content of cyanobacteria therefore appears to be environmentally modulated. The data presented here are the first evidence of environmental modulation of the BMAA content of cyanobacteria and, as such, offer the first insight into potential risk management approaches for waters prone to cyanobacterial blooms. Combined nitrogen availability, or the resulting cellular nitrogen status, had the greatest effect on the free cellular BMAA content in the strains tested. Specifically, nitrogen deprivation resulted in an increase in free cellular BMAA. Exposure to high light intensities (> 18 µmol m^{-2} s^{-1}) shortened the time required for medium combined nitrogen deprivation to result in increased BMAA. Furthermore, light quality affected the rate of BMAA production, with white light being more effective than blue light and both white and blue light eliciting a greater response than red light, at non-saturating intensities. Similarly, increasing phosphorous concentrations in media resulted in a slightly increased rate of production and quantity of free cellular BMAA. The physiological consequence of increased phosphorous is increased carbon fixation relative to nitrogen assimilation, at least in part due to a reduced glutamine synthetase reaction rate, with a consequential increase in glutamate:glutamine ratio.

Increased BMAA content as a function of environmental modulation appears to be transient, with BMAA concentrations reducing again over approximately the time frame required for the increase. This suggests either a regulatory role for BMAA or that it is a metabolic intermediate related to stress response physiology. This is supported by the effect of exogenous BMAA on *Synechocystis* (Downing et al., 2011) where BMAA caused nitrogen-deprivation type chlorosis even in the presence of combined nitrogen.

4.3 Production of BMAA Isotopes

Isotopically labelled BMAA was successfully synthesised using a proprietary method. The labelled products, 119.15 amu (BMAA-α^{15}N) and 123.34 amu (BMAA-4,4,4-d_3,$^{15}N_2$) were verified using LC/MS/MS.

4.4 Metabolism of BMAA by Cyanobacteria

As previously reported by Downing et al. (2012), extracellular BMAA is rapidly taken up by

cyanobacteria. The data presented here have also been discussed in WRC report 1885/1/13 in the context of exposure risk and environmental degradation or bioaccumulation of BMAA. However, the data are equally relevant to understanding possible biosynthetic routes in cyanobacteria.

Figure 4.4.1 shows the accumulation of isotopically labelled BMAA by *Synechocystis* PCC6803 over 48 hours and the concomitant increase in a BMAA with an *m/z* one less than the supplied BMAA, suggestive of atomic exchange. The redistribution of stable isotopes that must correlate with this was defined by a change, relative to control samples, in the abundance of the parent ion $^{x+}m/z$, where *x* equals 1 through 5.

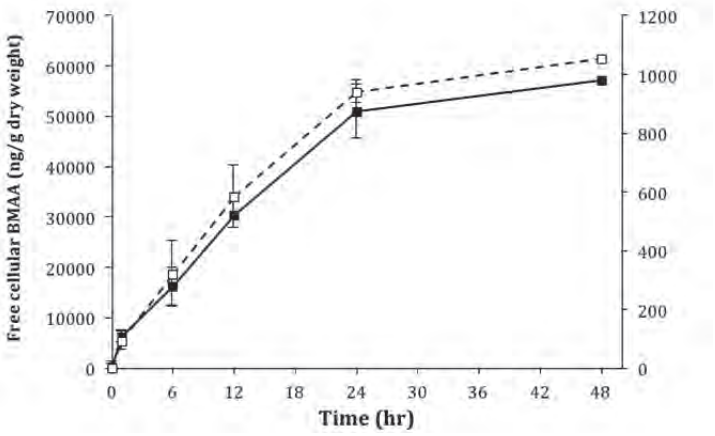

Figure 4.4.1. Free cellular $^{5+}$BMAA (black line, primary axis) and free cellular $^{4+}$BMAA (dashed line, secondary axis) over 48 hours in *Synechocystis* cultures exposed to 100 µM BMAA-4,4,4-d_3,$^{15}N_2$. Error bars denote standard deviation where n=3 (Downing et al., 2013).

Molecular isotopes of glutamate followed the same trend over the $^{+5}$BMAA exposure period, marked by a gradual and significant decrease in unlabelled Glu within the first 24 hours, followed by a significant ($p< 0.05$) increase in all detected glutamate molecular isotopes relative to the unlabelled Glu. This could be interpreted as either atomic exchange on the glutamate or continuous synthesis and loss of glutamate, with new glutamate being subject to labelling using the BMAA as a substrate.

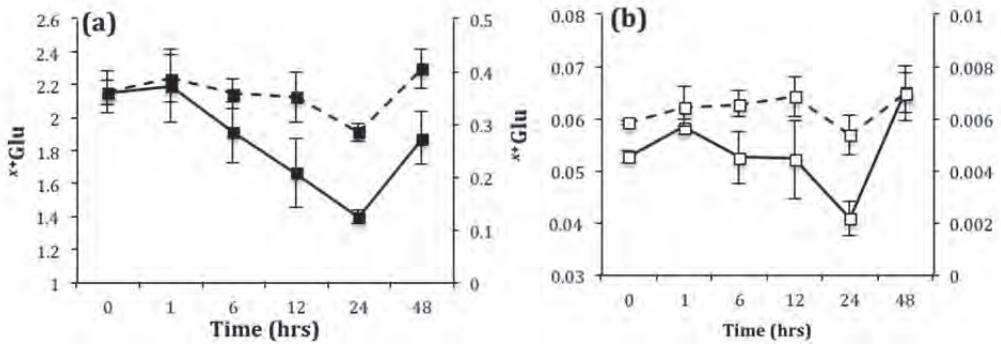

Figure 4.4.2. The change in free cellular glutamate over 48 hours exposure to BMAA-4,4,4-d_3,$^{15}N_2$, where (a) shows $^{0+}$glu (147.13 g.mol^{-1}) as a solid line and $^{1+}$glu (148.13 g.mol^{-1}) as a broken line (secondary axis); and (b) shows $^{2+}$glu (148.13 g.mol^{-1}) as a solid line and $^{3+}$glu (148.13 g.mol^{-1}) as a broken line (secondary axis). Error bars denote standard deviation where n=3 (Downing et al., 2013).

Results were normalised against the natural abundance of amino acid molecular isotopes which was determined based on amounts present at t0 (prior to the addition of BMAA-4,4,4-d_3,$^{15}N_2$). The data are presented in Figure 4.4.3 which shows the percentage change from t0 in glutamate stable isotopes relative to the entire free glutamate pool.

Although the total amount of $^{1+}$glu decreased over the initial 24 hours exposure, a significant increase in the relative abundance of $^{1+}$glu within the cell was observed over this period. The proportional increase in $^{2+}$glu and $^{3+}$glu was attributable to the natural abundance of stable isotopes receiving an additional ^{15}N.

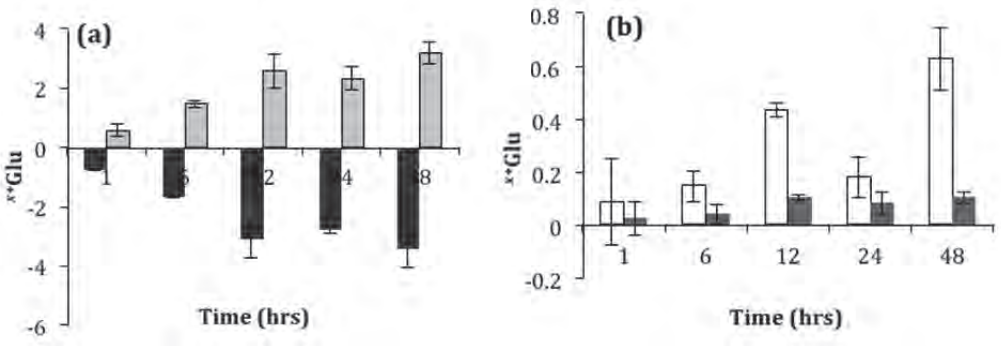

Figure 4.4.3 The change in free cellular glutamate isotopomers from their natural abundance at t0. (a) $^{0+}$glu (147.13 g.mol^{-1}) dark grey bars and $^{1+}$glu (148.13 g.mol^{-1}) light grey bars; and (b) $^{2+}$glu (148.13 g.mol^{-1}) open bars and $^{3+}$glu (148.13 g.mol^{-1}) light grey bars. Error bars denote standard deviation where n=3 (Downing et al., 2013).

Attributing the increase of $^{2+}$Glu and $^{3+}$Glu relative to $^{+0}$Glu, compared to that of $^{+1}$Glu relative to $^{+0}$Glu, to the natural abundance of either carbon or nitrogen isotopes in the amino acid pool, points to the transfer of the BMAA primary ammonia to 2-oxoglutarate by either transamination from, or deamination of, the labelled BMAA.

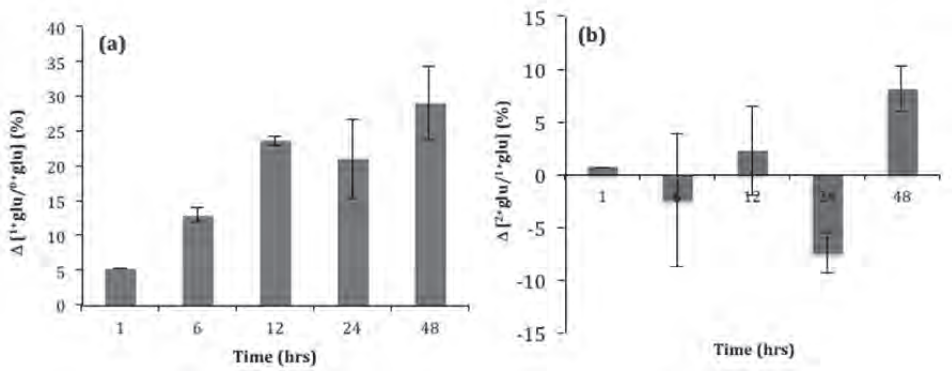

Figure 4.4.4. The change in free cellular $^{1+}$glu and $^{2+}$glu following exposure to $^{5+}$BMAA, expressed as a percentage change in the ratio of $^{1+}$glu: $^{0+}$glu (a) and $^{2+}$glu : $^{1+}$glu (b), respectively, with $^{x+}$glu at 0 hours taken as 100%. Error bars denote standard deviation where n=3.

Figure 4.4.4 shows this comparison of change in free cellular glutamate expressed as the percentage change in such ratios. The significant increase in $^{+2}$Glu relative to $^{+1}$Glu after 48 hours exposure indicates complete turnaround of the BMAA components.

If transamination is the final step in BMAA biosynthesis, it follows that the ammonium must not be added as free ammonia but rather from another amino acid such as the glutamate suggested above. If BMAA is deaminated and the available ammonia assimilated via glutamine synthetase (GS) or glutamate dehydrogenase (GDH), $^{+1}$Glu would result that in turn would result in $^{+2}$Gln as a function of addition of $^{+1}NH_4$ by GS.

Total free cellular glutamine increased over the 48 hour exposure period. Only a single glutamine isotope, $^{1+}$gln, was detected. Glutamine metabolism in cyanobacteria involves two enzymes; anabolic GS and catabolic, glutamine oxoglutarate aminotransferase (GOGAT). Glutamine synthetase catalyses the direct amination of glutamate by ammonium, thus acquiring its primary amino group from free cellular ammonia and its secondary amino group from glutamate. These data thus clearly show that BMAA nitrogen transfer to Glu was by transamination of 2-oxoglutarate since the presence of double-labelled Gln at levels exceeding natural abundance could only occur as a result of BMAA deamination. Furthermore, more than half of the glutamine pool became singly labelled.

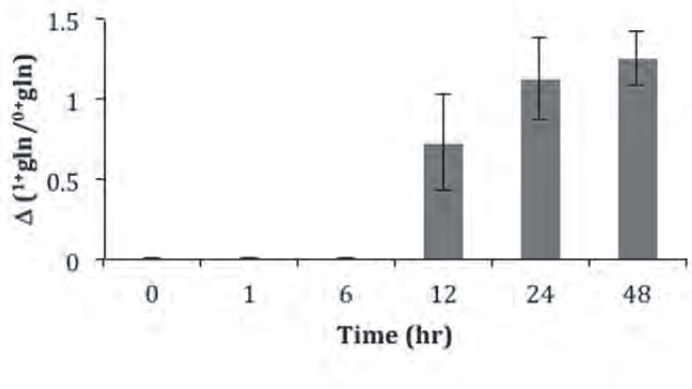

Figure 4.4.5. The change in free cellular $^{1+}$Gln following exposure to $^{5+}$BMAA, expressed as the change in the ratio of $^{1+}$gln: $^{0+}$gln, normalised against the ratio at t0. Error bars denote standard deviation where n=3 (Downing et al., 2013).

It is also important to note that $^{1+}$glu increased within the first one hour of exposure to $^{5+}$BMAA but by a fairly low percentage relative to the entire free glutamate pool. In contrast, $^{1+}$gln only increased after 12 hours exposure to $^{5+}$BMAA with a total 56% increase relative to the entire glutamine pool over the entire exposure period. This suggests that the continued assimilation of ammonia resulted in the continued increase in Gln from labelled Glu.

The most likely metabolism of BMAA is therefore transamination reactions involving primary amino groups. These are very simple, common and reversible in cyanobacteria. Their reversibility demands the appearance of $^{+4}$BMAA, since the produced keto acid should similarly act as substrate for transamination from an amino acid, as observed.

4.5 Alternatives to Measuring BMAA

Analysis of BMAA depends on an expensive and precise method requiring sophisticated equipment. This makes routine monitoring difficult and impractical. In an attempt to identify a cheaper and simpler indicator of possible BMAA presence, co-metabolites of BMAA were sought. The disappearance of free cellular BMAA upon addition of ammonia to nitrogen-depleted cultures has been repeatedly shown. The previously reported BMAA amination product data and feeding experiment data suggested that free cellular BMAA has a highly transient nature with it being produced, at least partially, as a function of deamination of aminated carbamates and removed due to amination of carbamates of BMAA. This offers the possibility that other amino acids may serve as indicators of BMAA presence. Amino acids analyzed (Table 4.5.1) were chosen based on the nitrogen modulation of BMAA and amino acid feeding.

4.5.1 Correlation of BMAA with amino acids

A controlled experiment was conducted in which cultures were transferred from a combined nitrogen-free medium to medium containing ammonia and nitrate; trends in the concentrations of amino acids of interest were observed. The results are shown in Figure 4.5.1.

Table 4.5.1. Amino acids used in this study showing retention time (t_R) and molecular weight (MW).

Amino acids	t_R (min)	Amino acid MW
Arg	2.7	174.2
Gln	3.3	175.2
Ser	3.7	105.1
Asn	3.8	132.1
β-Ala	5	89.1
Ala	5.1	89.1
Sar	5.8	89.1
2,4-DAB	6.7	118.1
Orn	6.9	132.1
BMAA	7.4	118.1
Lys	7.8	146.1
Asp	7.8	133.1
Val	8.2	117.1
Glu	8.3	147.1

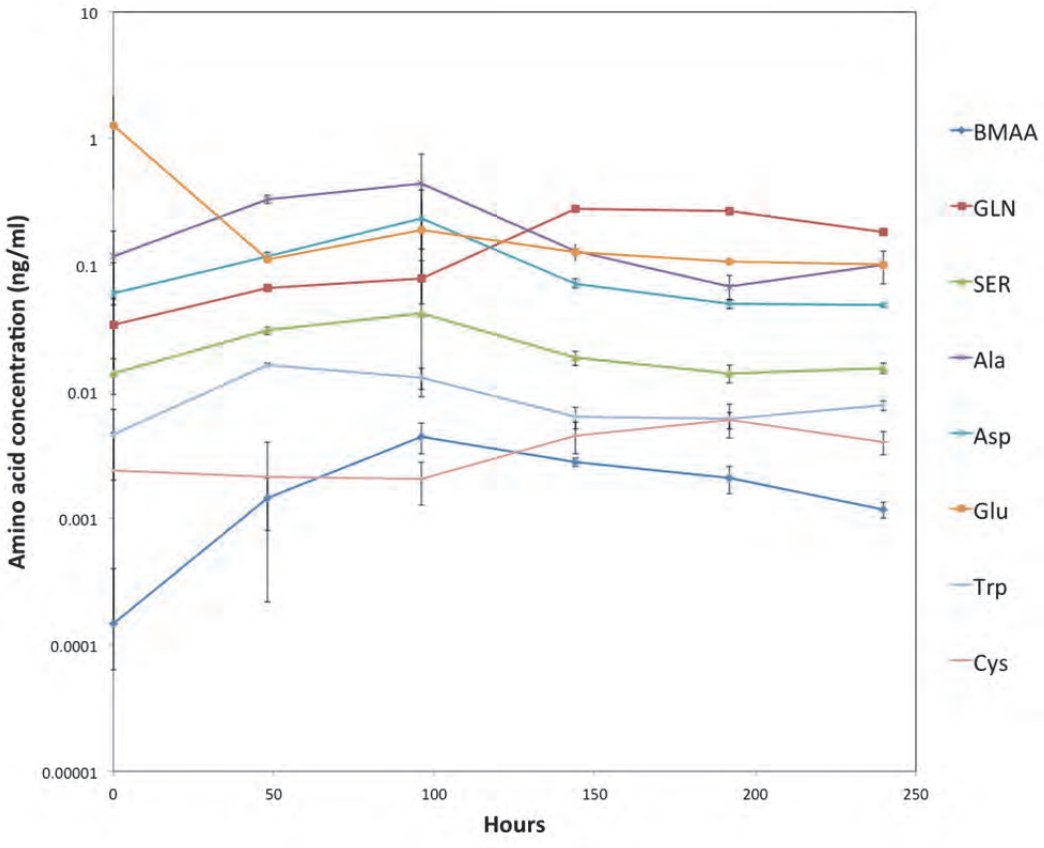

Figure 4.5.1. Variation in amino acids over a ten day period in nitrogen-containing medium. Error bars represent standard deviation (n=3).

Although relatively good correlations appear to exist for certain amino acids (both serine and alanine had correlation coefficients with BMAA of 0.607), over a ten day period these were not statistically significant as tested with a t-test. Glutamate, however, during a deprivation-feeding-deprivation

regime, had a significant (p=0.001) correlation of 0.959 (see Figure 4.6.2) emphasizing the relationship between glutamate and BMAA and the influence of cellular nitrogen status on BMAA content. Furthermore, since this relationship was obtained both during nitrogen assimilation from a physiological status of nitrogen deprivation and in the following deprivation phase of the experiment, this correlation may be environmentally robust.

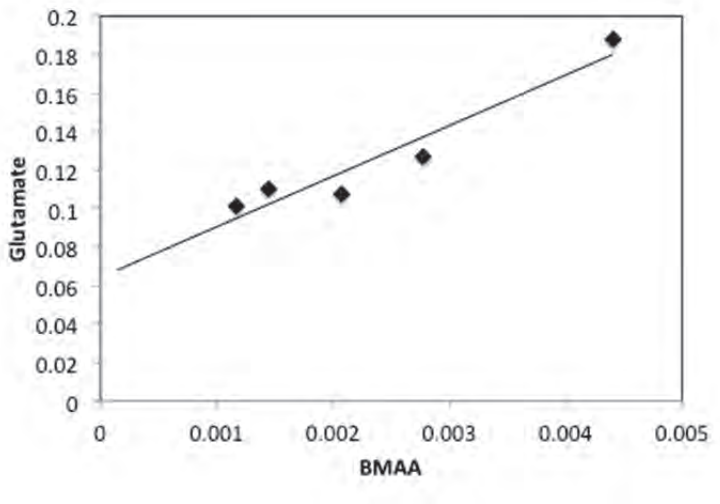

Figure 4.5.2. The relationship between glutamate and BMAA during growth on nitrogen-replete medium following nitrogen starvation, and during subsequent starvation.

If the good correlation with glutamate is based on carbon:nitrogen balance, then this should strengthen when the Glu:Gln ratio is plotted against BMAA. This was, however, not the case during nitrogen replete growth. Although this is quite informative in terms of establishing metabolic mechanisms for BMAA in that glutmate is metabolically intimately linked to BMAA, it would appear to make the use of both C:N ratio or glutamine:glutamate ratio, as well as either of these amino acids singly, of little value as predictors of BMAA content. Since most easily measured metabolites or structural components of the cyanobacterial cell are modulated or regulated by the C:N status of the cell, these also become useless for prediction or estimation of cellular BMAA content. Furthermore, the complexity introduced by more complex environments further negates the use of any nitrogen or carbon primary metabolite, as is explained in section 4.2. Long-term monitoring and modelling of environmental parameters affecting BMAA concentration may resolve this complexity at least in as far as developing indicators of potential BMAA levels of concern.

4.6 BMAA Production During Metabolic Manipulation By Feeding

Evaluation of metabolic manipulation in order to simulate nitrogen deprivation was accomplished by culturing in α-ketoglutarate as described above. A significant increase in BMAA content was observed (Figure 4.6.1) presumably due to the scavenging of cellular ammonia and the subsequent nitrogen-starvation response in the cyanobacteria, while at the same time increasing the Glu pool and thus making amino groups available for the postulated transamination of the BMAA keto acid 2-oxo-3(aminomethyl)propanoic acid.

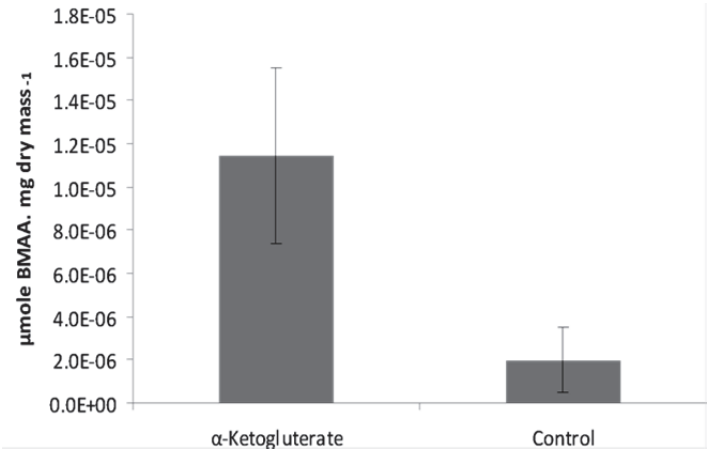

Figure 4.6.1. Free cellular BMAA concentrations in cultures of *Synechocystis* grown in the presence and absence of 1 mM α-ketoglutarate for 8 days without any combined nitrogen in the medium. Error bars are standard deviation (n = 3).

These data support nitrogen deprivation and cellular carbon:nitrogen balance as modulators of BMAA content in cyanobacteria and offer a possible physiological state as an indicator of BMAA presence. Nitrogen starvation typically results in observable changes in pigment ratios with the reduction of phycobilliprotein content relative to chlorophyll*a*. This is particularly interesting since exogenous BMAA rapidly results in cyanobacterial bleaching.

There was a slight but insignificant increase in BMAA after 24 hours in cultures fed aspartate. Significantly increased BMAA content was observed after 120 hours in cultures grown on tryptophan and cysteine as sole nitrogen source, as is clearly visible in Figure 4.6.2c; whereas after 24 hours, Trp yielded a significantly reduced BMAA content. After 120 hours the BMAA produced as a function of nitrogen deprivation had returned to typical nitrogen-replete levels as a function of nitrogen re-distribution from the supplied amino acids, emphasizing the transient nature of BMAA in the cell. The increase in BMAA observed in the cultures that were fed tryptophan and cysteine suggests a metabolite of these amino acids as a potential precursor to BMAA.

It was expected that all tested amino acids (Ala, Asp, Cys, Ser and Trp) would initially, although transiently, increase available free cellular ammonia concentrations due to potential deaminations or transaminations of primary or secondary amines and thereby initially prevent/reduce BMAA production. This is reflected in the initial delay in BMAA synthesis in the presence of all amino acids tested, with the exception of Asp, as seen in Figure 4.6.2.a. This may indicate an oxaloacetate-aspartate transaminase as a possible enzyme for the transamination of the BMAA keto acid with Asp as substrate.

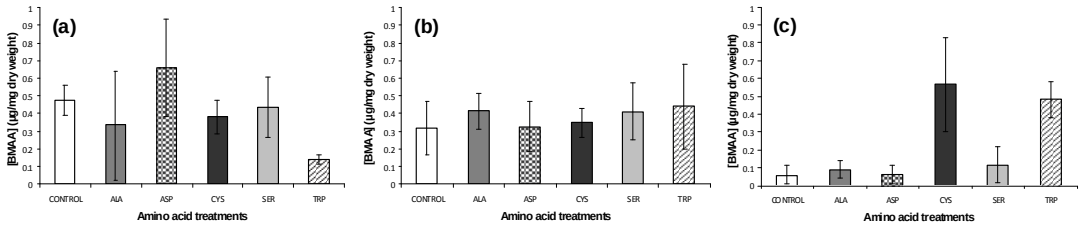

Figure 4.6.2. Free cellular BMAA content of *Synechocystis* J341 cells following (a) 24 hours, (b) 72 hours and (c) 120 hours growth in modified $BG11_0$ media (containing no nitrogen) supplemented with a single amino acid (1mM). Control treatments were not supplemented. Error bars denote standard deviation where n=3.

BMAA production and its presence within cells appears be transient in control cultures as well as those supplemented with Ala, Asp, and Ser. However, free cellular BMAA increased steadily in the presence of Trp and Cys, beyond the transient peak observed in control cultures. The possibility remains that the Trp-induced and Cys-induced delay might follow the same transient pattern but over a longer period; however, the delay based on availability of ammonium from deamination would not be any different for these two amino acids. Alternatively, these data could suggest that Cys and Trp metabolites are precursors to a common molecule that is required in BMAA biosynthesis, or that elevated levels of these two amino acids are associated with cellular states under which BMAA is produced. This makes these amino acids good candidates for co-metabolites. Cys and Trp catabolism yields pyruvate; however, this molecule is not specific to the catabolism of only these two amino acids, but is also an end product of the catabolism of other amino acids, including Ser and Ala that did not give the same result. In bacteria, Trp is a precursor to niacin, a precursor of NAD and NADP and plays a role in the activation of heterocyst differentiation in diazotrophic cyanobacteria (Bottomley et al.,1980). Cys is required for the synthesis of glutathione, a tripeptide involved in cellular protection against oxidative stress and other environmental stresses (Zechmann et al., 2010). This is essential in cyanobacteria that can produce large amounts of reactive oxygen species (ROS) during aerobic photosynthesis. An increased production of free cellular BMAA in the presence of elevated cellular Cys concentrations may be due to an association between high levels of Cys and subsequent increased glutathione production, mimicking a cellular state characteristic of a cell under oxidative stress. Oxidative stress, and cellular states characteristic of oxidative stress, is also common under conditions of nitrogen limitation, which results in BMAA production in cyanobacteria (deliverable 1 report). This is due to an imbalance between light-dependent electron transport and carbon fixation-driven electron consumption, which results in an increase in reactive oxygen species, as during nitrogen limitation, the rate of carbon fixation decreases. However, since BMAA either elicits a response that results in restoration of the pre-BMAA state, or is produced in response to a stress state in an attempt to restore the pre-stress state, the stress state is not in itself usable as a BMAA indicator, particularly since the general stresses induced by BMAA, or resulting in its production, may occur as a result of exposure to other molecules or environmental states. The continued increase in BMAA with tryptophan and cysteine feeding is of interest, though, primarily because the previously

noted negative correlations between BMAA and tryptophan suggest a tryptophan metabolite as a BMAA precursor.

The apparent contradiction of BMAA and DAB co-occurrence in starved cultures and the absence of BMAA in serine-fed cultures can be explained in the context of tryptophan-induced BMAA increase. The formation of indole and serine from excess tryptophan coincides with an increase in BMAA and DAB whereas the increase in DAB in the presence of serine does not occur with a corresponding increase in BMAA. Thus it is specifically the breakdown of Trp that must induce BMAA synthesis or result in covalent modification of a substituted BMAA. Thus tryptophan may serve as an indicator of BMAA absence. Similarly, tryptophan catabolism can yield alanine that also shows a positive correlation with free cellular BMAA when cultures are fed on Cys as a nitrogen source, although alanine feeding does not increase BMAA content and in the absence of external organic nitrogen the correlation with glutamate disappears. This further suggests that tryptophan degradation may provide precursor molecules for BMAA synthesis. The reasonably good correlation between cellular Gln and BMAA in cultures grown on Trp-containing medium also suggests that Trp degradation yielded amino groups for Gln synthesis and provided the necessary precursors to BMAA since Gln and BMAA should be inversely related given the production of BMAA under nitrogen stress. That this does not appear to be the case suggests that glu:gln ratios, or indeed C:N ratios, would not be useful in estimating BMAA content.

These data suggest a potential mechanism for BMAA estimation based on the ratio of tryptophan to another amino acid, probably glutamate or glutamine. However, the removal of the glutamate–BMAA correlation in the presence of cysteine in particular (see below) makes this an unreliable candidate for BMAA estimation based on co-metabolites, not least because it would require both photometric and enzymatic assays to establish these ratios.

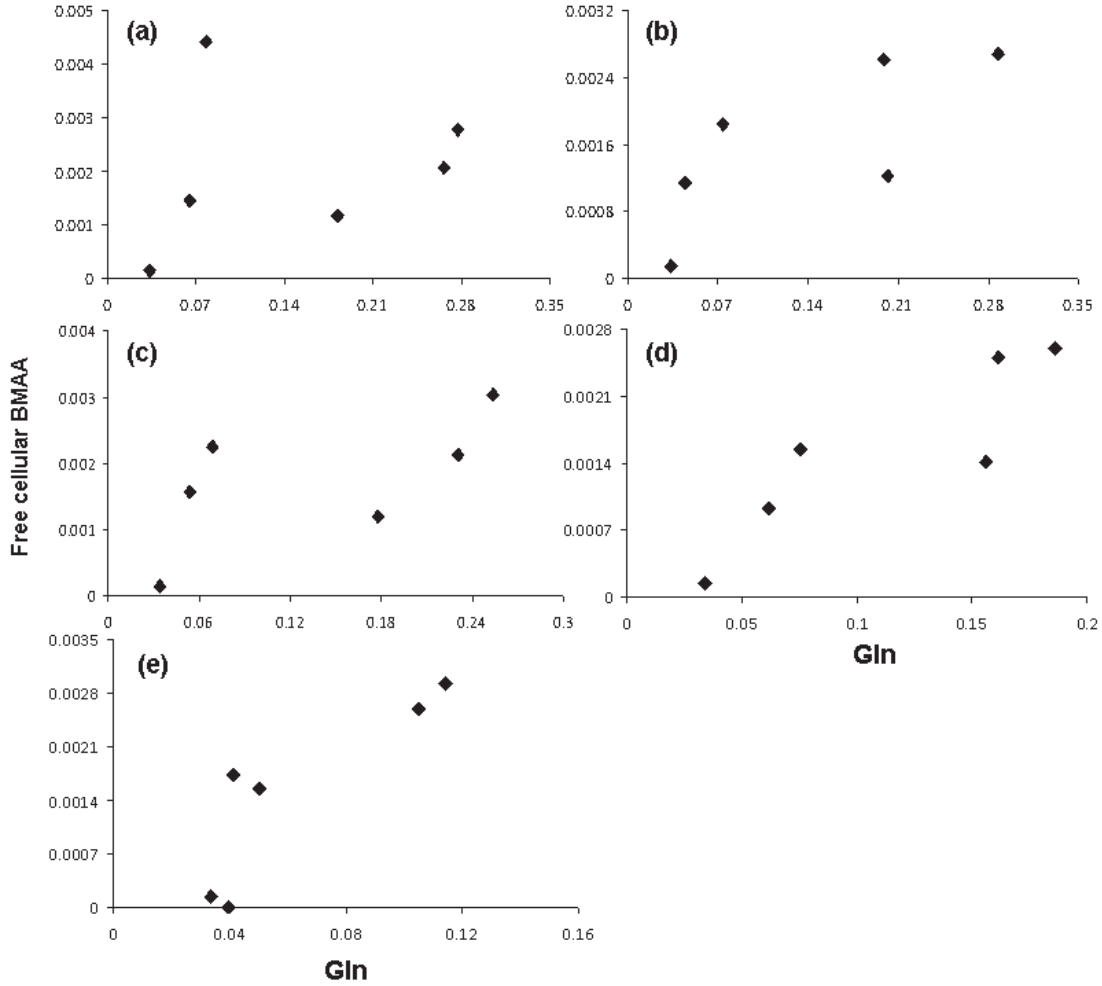

Figure 4.6.3. Positive correlations between an increase in free intracellular glutamate and free cellular BMAA production in *Synechocystis* cultures grown in (a) the absence of combined nitrogen, or in the presence of (b) Ala; (c) Asp; (d) Ser; (e) Trp, respectively, as sole nitrogen source. BMAA and Gln are represented as LC/MS peak areas of detection of parent ions 333 *m/z* and 275 *m/z* respectively, relative to that of the internal standard, Methionine-D3, 317 *m/z.*

The relationship between Glu and BMAA that occurs in control cultures and in cultures grown on Asp (Figure 4.6.3) or other Glu transamination products does not hold true for Cys which actually results in a negative relationship between Glu and BMAA. This makes this relationship dependent on the external organic matrix and thus unreliable as an indicator of BMAA content.

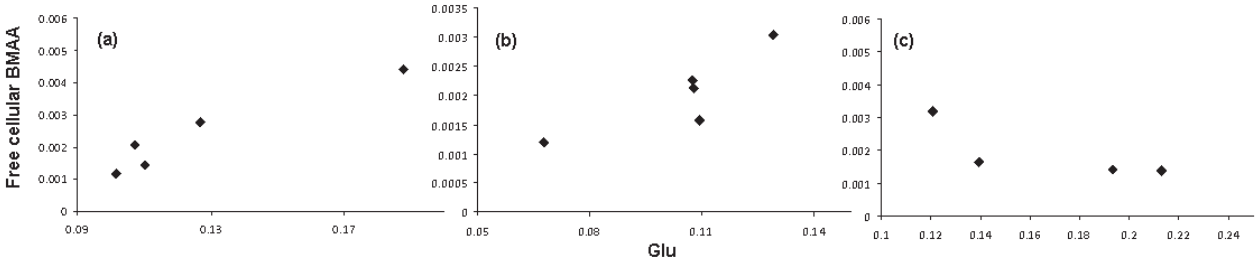

Figure 4.6.4. Relationship between free cellular glutamate and free cellular BMAA in *Synechocystis* cultures grown in (a) the absence of nitrogen; and in the presence of (b) Asp; (c) Cys; as sole nitrogen source, respectively. BMAA and Glu are represented as LC/MS peak areas of parent ions 333 *m/z* and 318 *m/z,* respectively, relative to that of the internal standard, Methionine-D3, 317 *m/z.*

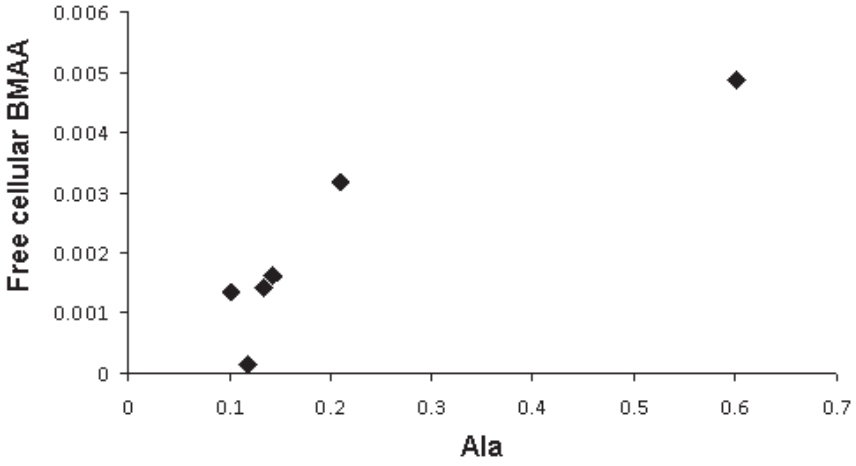

Figure 4.6.5. A positive correlation between free cellular BMAA and free cellular alanine in *Synechocystis* cultures grown in the presence of cysteine as sole nitrogen source. BMAA and alanine are represented as LC/MS peak areas of parent ions 333 *m/z* and 218 *m/z*, relative to that of internal standard Methionine-D3, 317 *m/z.*

Not only does Cys as a sole nitrogen source destroy the relationship between Glu and BMAA, it also creates a positive relationship between Ala and BMAA and results in increased BMAA, or at least BMAA increase over a longer period than the transient control increase. Given the transience of BMAA under environmental conditions and the variability in correlations between BMAA and other metabolites as a function of external organic matrix components, it seems unlikely that a directly correlated and easily measurable co-metabolite will be found. In the absence of an observable physiological indicator state or measurable co-metabolite, the toxin itself must be measured to ensure safety, or a model based on environmental parameters and supported by physiological and functional

data needs to be developed. As the currently acceptable methods are technically demanding and require expensive equipment, an alternative is required. The alternative need only provide an indication of potential BMAA presence, which can then be confirmed by the more sophisticated MS or MS/MS methods.

4.7 The Effect of Growth Phase and Growth Rate on BMAA Production

Growth rate is not an effector of BMAA production. At all positive growth rates tested in the laboratory, whether in batch or continuous culture (chemostat), no significant quantities of BMAA were observed in the known *Synechocystis* producer J341. At nitrogen-limited steady state, at growth rates of 0.1 x μ_{max}, 0.45 x μ_{max} and 0.86 x μ_{max}, BMAA levels were insignificant.

In batch culture with nitrogen limitation, BMAA was observed after prolonged starvation as described above. The homeostasis that occurs even under continuous nitrogen limitation is such that at steady state the cells have adapted their carbon fixation and the apparent C:N balance. This further supports the hypothesis that BMAA is a stress response regulator as this type of molecule would not be present in a steady state population adapted to a particular nutrient regime.

4.8 ELISA

The quantitative accuracy of the supplied standard curve is depicted in Figure 4.8.1. Inaccurate standards were present in three out of three kits tested. Figure 4.8.2 shows the standard curve prepared with Sigma BMAA standard and analysed according to the resulting curve to yield a usable reference.

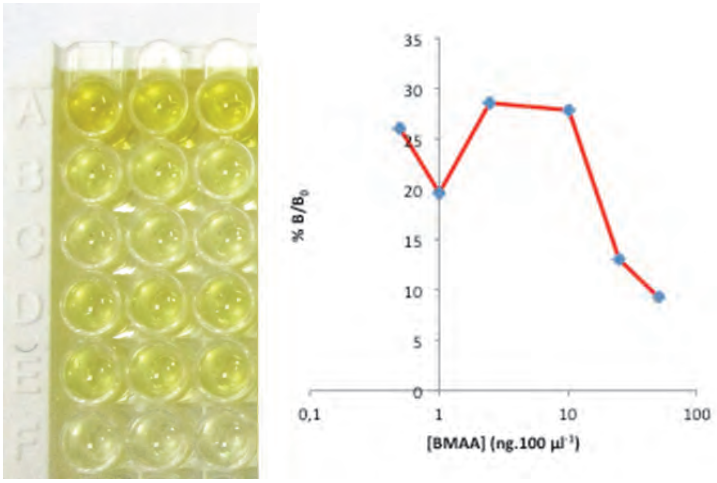

Figure 4.8.1. The ELISA plate showing the relevant wells containing the supplied standards in triplicate (Left) and the resulting standard curve (Right) as measured using the supplied standards.

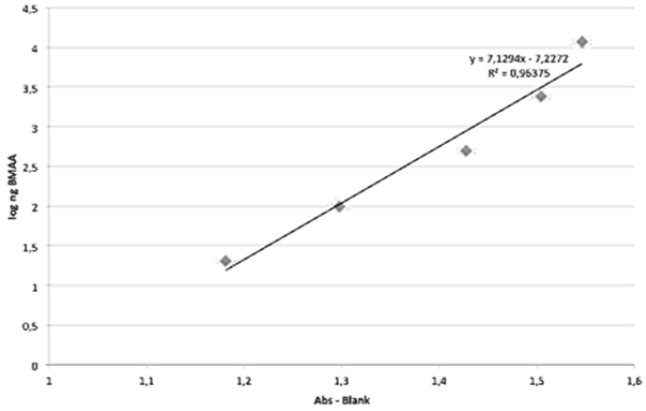

Figure 4.8.2. A standard curve of separately purchased BMAA (Sigma) plotted as the log of the BMAA against the difference in the absorbance between the test sample and the blank. Points represent averages of three replicates.

The accuracy of the kit in a variety of matrices is shown in Figure 4.8.3. In many cases the kit overestimated the BMAA concentration substantially. In particular, the presence of an amino acid matrix at 200 µM resulted in a very large overestimation of the BMAA concentration. This strongly suggests non-specificity in the antibody used. The effect is more pronounced in matrices of biological tissues as shown in Figure 4.8.4.

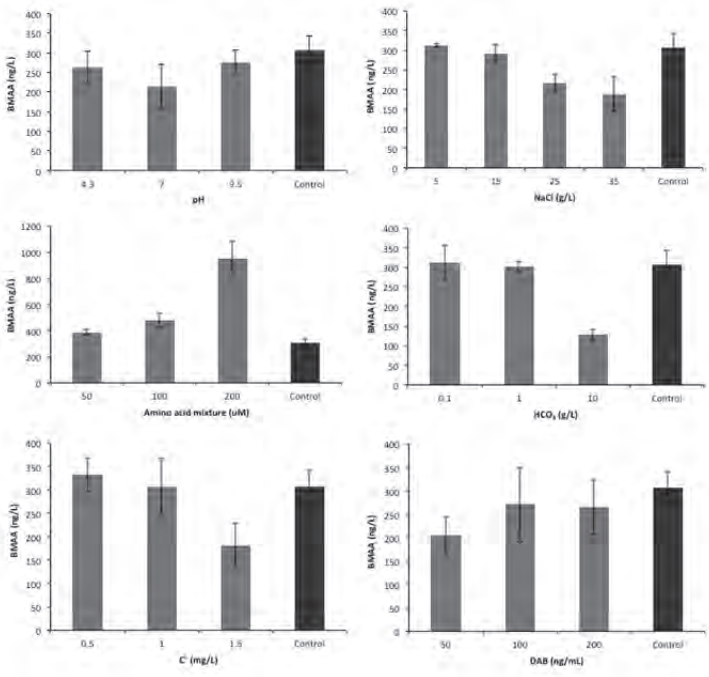

Figure 4.8.3. 300 µM BMAA quantification in a variety of matrices relative to a control of BMAA in water. Controls are all BMA in deionized water. Error bars represent standard deviation (n=3).

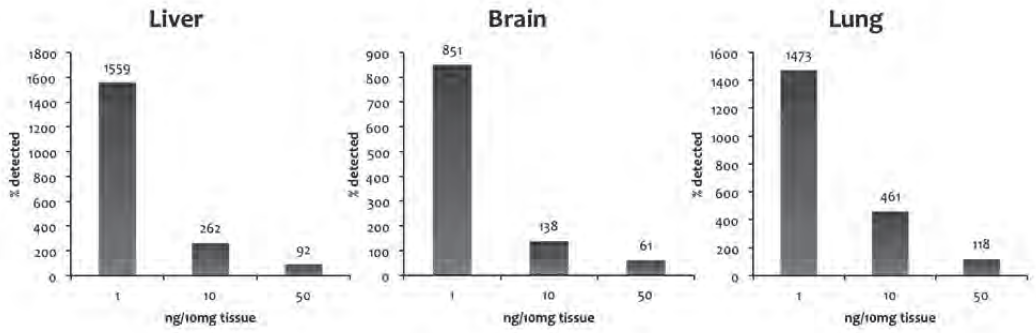

Figure 4.8.4. BMAA estimation by ELISA in complex biological matrices showing overestimation in most cases.

BMAA is a transient metabolite that appears to play a role in transitioning between cellular carbon:nitrogen states. As the metabolome is generally in flux during extreme C:N variations it seems unlikely that a co-metabolite that is not a direct precursor or product of BMAA will be found. Furthermore, the direct precursors and products appear to form part of complex branching metabolic pathways and as such may vary differently to BMAA under different environmental conditions. In the light of these data it seems prudent to continue environmental monitoring and establishment of a large dataset of environmental variables and BMAA concentrations so as to begin to develop a predictive model based on environmental parameters. In the interim, the ELISA kit may be used as an indicator, but the lack of accuracy and the requirement for cellular lysis and analysis in the absence of a complex matrix make this method unsuitable for general use as a definitive measure of BMAA in bloom samples.

5 CONCLUSIONS

BMAA is produced under nitrogen-stress conditions either as a function of combined nitrogen deprivation due to low medium-nitrogen levels, or due to increased levels of carbon fixation metabolites which in turn results in reduced cellular ammonia. Any parameter influencing the apparent cellular nitrogen availability, such as increased light intensity, increased phosphorous, or the availability of excess amino acids that predominate under nitrogen stress, results in increased BMAA. In the case of heterocystous diazotrophic species, the mechanism of distribution of nitrogen to vegetative cells from heterocysts can result in similar cellular conditions.

The complexity of metabolism associated with BMAA, and the metabolic C:N homeostasis mechanisms within cyanobacteria, make it extremely difficult to identify a single co-metabolite or a set of co-metabolites that correlate with BMAA. This, in conjunction with the transient nature of BMAA, strongly suggests that BMAA is a response regulator for nitrogen deprivation. This is supported by the published effect of internalised exogenous BMAA on cyanobacteria where the cell responds in a manner consistent with nitrogen deprivation, even in the presence of nitrogen (Downing et al., 2012).

BMAA analysis requires expensive and sophisticated equipment. The cost of analysis is such that routine monitoring for BMAA in surface waters is not feasible using current, published methods. The lack of a co-metabolite or detectable cell status that can be easily and affordably measured makes it essential to determine whether the observed physicochemical conditions that lead to BMAA production in the laboratory, specifically nitrogen stress, can be correlated with BMAA in natural bloom samples. This would allow the development of a model to inform an alert level framework for accurate quantitation of BMAA when necessary.

Metabolite feeding and pulse chase analysis data suggest that the last phase of production of BMAA is either via transamination of a keto acid of BMAA, or via removal of the carbamate of one or both of the two possible carbamate molecules. Furthermore, amination of the spontaneously formed carbamates was demonstrated and we therefore propose a mechanism based on linearisation of a cyclised aminated carbamate product of BMAA with subsequent deamination and spontaneous decarbamation to yield BMAA. We also speculate that the predominant carbamate of BMAA, the α-carbamate, may be the response regulator in cyanobacteria.

Any environmental model for BMAA production requires a scientific basis in order to ensure that it is sufficiently robust. Confirmation of the regulatory function of BMAA in cyanobacteria is therefore necessary to validate the proposed environmental model that would result from a multi-season monitoring programme.

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