The occurrence and environmental fate of cyanobacterial β-N-methylamino-L-alanine and consequential potential human exposure

A report emanating from the project entitled:

β-N-methylamino-L-alanine Bioaccumulation and Biomagnification: Health Risks and Treatment Possibilities

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

Background and motivation for research

β-N-methylamino-L-alanine (BMAA) is a neurotoxic amino acid produced by cyanobacteria. BMAA has been implicated in neurodegenerative disease as it causes motor neuron damage at fairly low exposure concentrations. Potentially harmful BMAA within cyanobacterial cells may be released on cell senescence. Collapse of a substantial cyanobacterial bloom may result in release of large amounts of the toxin into water. The fate of that toxin, at the time of initiation of this project, was unknown. In order to evaluate potential risk to consumers, the fate of the toxin in the environment, the environmental consequences of BMAA in the water, and the potential for contamination of drinking water needed to be determined.

Project objectives

The purpose of this project was to establish the potential for human exposure risks from ingestion of BMAA via water and food supplies contaminated with BMAA of cyanobacterial origin. Such exposure may occur via direct consumption of free BMAA in water supplies or via bioaccumulated BMAA in animal or plant tissue. However, the potential for such bioaccumulation in freshwater aquatic systems was unknown. This project therefore aimed to identify the potential for bioaccumulation in different freshwater aquatic model organisms as well as the potential for biomagnification in potential human or animal food resources. Subsequent to our reports on typical water treatment efficacy and the rapid uptake of BMAA by, and ecotoxicological effects on a wide range or organisms, the project was amended to include confirmation of the cyanobacterial origin of BMAA and attempt to elucidate possible biodegradative fates of BMAA. The total knowledge contributions would therefore be in human health risks, environmental health and phytoremediation potential for BMAA removal. To these ends the following were specific project aims:

- To determine whether BMAA bioaccumulates in cyanobacterial grazers and other aquatic organisms
- To determine whether bioaccumulated or unincorporated BMAA can be bioaccumulated by predators of cyanobacterial grazers
- To monitor toxicological effects of BMAA on all these organisms in order to evaluate their use as biosensors.
- To evaluate biomagnification in selected food chains.
- To evaluate standard treatment methods for BMAA removal
- To determine the suitability of bioaccumulation of BMAA as a treatment option and compare it to existing water treatment methods
- To provide recommendations and guidelines for BMAA monitoring and water treatment for BMAA removal if necessary

These aims were initially to be addressed by completing the following deliverables:

- Report on the evaluation of bioaccumulation in model organisms
- Report on the evaluation of BMAA removal by standard water treatment methods
- Report on BMAA biomagnification potential in aquatic ecosystems
- Report on the toxicological effects of bioaccumulated BMAA
- Report on the potential of bioaccumulation as a water treatment option

However, results obtained and published during this project received some criticism in the light of the analytical debate surrounding BMAA. Specifically, several laboratories had been unable to detect BMAA in cyanobacterial isolates. Although there are several reasons for this (see Banack et al., 2010) some scepticism remained and impacted on the acceptance of ecotoxicology data. It was therefore recommended by the project steering committee that the de novo production of BMAA in cyanobacteria be confirmed and that isotopically-labelled toxin be produced for confirmation of uptake and potential metabolism of BMAA. This new deliverable replaced the deliverable “To determine the suitability of bioaccumulation of BMAA as a treatment option and compare it to existing water treatment methods” which was to be accomplished by the evaluation of a ponding system for phytoremediation which, given the rapid uptake already demonstrated by several organisms, was deemed less important without confirmation of toxin source, toxin uptake, and potential information on biotransformation.

Methodology

In order to assess the risk of exposure of humans to BMAA from freshwater sources, the efficacy of standard water treatment processes was determined at laboratory scale. Additionally, the potential dissemination of the toxin in aquatic food webs was evaluated along with the potential for exposure directly from ingestion of treated water. Specifically, the cyanobacterial origin of BMAA was confirmed by stable isotope feeding of an axenic culture. This was done concurrently with research done in collaboration with the Institute of Ethnomedicine in the USA to address certain analytical questions and in so doing produce irrefutable evidence of the cyanobacterial origin of BMAA. BMAA uptake was studied in a range of organisms by controlled exposure to various concentrations of BMAA and determination of BMAA content and where relevant, distribution in the exposed organism. An example of transfer through an aquatic ecosystem was also studied by quantifying the accumulation and transfer of BMAA from catfish to crocodiles. The effect of the BMAA on model organisms was also investigated by determining their stress responses to exposure. These data provided sufficient understanding to suggest the feasibility of phytoremediation but did not adequately determine the potential for biotransformation. Molecular isotopes of BMAA were therefore synthesized and these used to evaluate uptake and biotransformation by cyanobacteria, an aquatic macrophyte and aquatic invertebrates.

Summary of the major results and conclusions

BMAA was rapidly taken up in its free form by a range of organisms including cyanobacteria, the aquatic macrophyte *Ceratophyllum demersum* (common hornwort), the terrestrial plants *Daucus carota* subsp. *sativus* (carrot) and *Nasturtium officinale* (garden cress), the invertebrate *Daphnia magna* as well as the mussels *Dreissen polymorpha* (zebra mussel), *Anadonta signia, Unio pictorum* and *Caubicula javanicus*. Furthermore, BMAA was observed in African sharptooth catfish (*Clarius garipinus)* and crocodiles (*Crocodylus niloticus)* that had been feeding on the catfish. These data suggest that BMAA is rapidly taken up by a wide range of organisms and becomes freely available in food webs. Furthermore, our data show that bioaccumulation occurs in certain species while biotransformation can occur in some species. Insufficient data were obtained to confirm biomagnification in the one aquatic food chain tested but what limited samples were available suggested no biomagnification although transfer through food webs to organisms that pose a potential human exposure risk is likely. The variability in uptake, accumulation and biotransformation by different organisms suggest that, despite the likelihood of generalized exposure risk, individual ecosystems would have to be investigated at a component level to fully understand the environmental fate and potential human exposure risk in those systems.

The rapid uptake of free BMAA from water by many organisms explains the absence of free BMAA in any raw water tested. Nonetheless, the potential for release of large amounts of free BMAA on cyanobacterial bloom collapse necessitated the evaluation of standard water treatment methods for BMAA removal. Standard methods including sand filtration, chlorination and the use of activated carbon were all successful at removing BMAA at laboratory scale. The absence of BMAA in any treated water tested, including treated water from bloom-containing raw water, confirms that standard water treatment practices adequately protect consumers from BMAA at known concentrations in raw water. However, the presence of cyanobacterial cells in drinking water remains cause for concern given the intracellular nature of BMAA.

All tested organisms displayed typical stress responses on exposure to exogenous BMAA. This may be significant in terms of cyanobacterial hepatotoxin exposure where such an effect may result in synergistic toxicity and enhance the effect of the hepatotoxins.

Recommendations

Given the potential for human exposure demonstrated here, it is recommended that the actual human exposure be evaluated at selected sites and that specific ecosystems be studied with respect to the biotransformation and/or bioaccumulation of BMAA. No specific steps to reduce possible exposure risk are, as yet, indicated. The practice of posting warning notices in the event of cyanobacterial blooms should continue in the absence of knowledge on aerosol exposure and actual risk of ingestion of fish and/or other aquatic organisms. These risks should be evaluated in a study of selected impoundments prone to cyanobacterial blooms, wherein aerosol exposure by nearby residents, recreational users

and any commercial activity be evaluated. Linked to this, an epidemiological study should be initiated where a food item shown to accumulate BMAA and sourced from that impoundment is usually available, and the transfer of BMAA to consumers can be evaluated.

Capacity development

The following students completed their degrees working on this project:

- Dr Maranda Esterhuizen – PhD
- Mr Fransois Niyonzima – MSc
- Mr Raymond Krisman – BSc (Hons)
- Mr Patrick Mwanza – BSc (Hons)

Ms S Downing (nee Searle) 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 after the completion of K5/2065.

REFERENCE GROUP

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LIST OF ABBREVIATIONS

ABTS: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

AD: Alzheimer's disease

ALS: amyotrophic lateral sclerosis

AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

amu: atomic mass unit

ANOVA: analysis of variance

APx: ascorbate peroxidase

AQC: 6-aminoquinolyl-N-hydrosuccinimidyl carbamate

BMAA: *β-N*-methylamino- L-alanine

BOAA: α-amino-β-oxalylaminopropionic acid

BSA: bovine serum albumin

CAT: catalase

CDL: curved desolvation line

cAMP: cyclic adenosine 5'-mono-phosphate

CDNB: 1-chloro-2,4-dinitrobenzene

DAB: 2,4-diaminobutyric acid

DNA: deoxyribonucleic acid

DTE: dithioerythritol

DW: dry weight

EDTA: ethylenediaminetetraacetic acid

ESI: electrospray ionization

Fig.: figure

FD: fluorescent detection

FMOC: fluorenylmethyloxycarbonyl

GC: gas chromatography

GPx: glutathione peroxidase

GR: glutathione reductase

GSH: reduced glutathione

GSSG: glutathione disulfide (oxidized glutathione)

GST: glutathione *S*-transferase

HARG: homoarginine

HILIC: hydrophobic interaction chromatography

HPHE: homophenylalanine

HPLC: high-pressure liquid chromatography

hr(s): hour(s)

ISTD: internal standard

kat: katal

ℓ: liter(s)
LC: liquid chromatography
LLOD: lower limit of detection
LLOQ: lower limit of quantification
M: molar
MC: microcystin
MDHA: monodehydroascorbate
Met-D3: methionine D3
mGluRs: metabotropic glutamate receptors
min: minute(s)
mol: moles
MS: mass spectrometry
MS/MS: triple quadropole mass spectrometry
m/z: mass charge ratio
$NADP^+$: oxidised nicotinamide adenine dinucleotide phosphate
NADPH: reduced nicotinamide adenine dinucleotide phosphate
ND: not detected
nmol: nanomoles
NQ: not quantifiable
NMDA: *N*-methyl-D-aspartate
PAC: powdered activated carbon
PCR: polymerase chain reaction
PD: Parkinson's disease
PDC: Parkinsonism dementia complex
POD: peroxidases
PSP: progressive supranuclear palsy
ROS: reactive oxygen species
rRNA: ribosomal ribonucleic acid
RSD: relative standard deviation
RT: retention time
sec: second(s)
SOD: superoxide dismutase
SD: standard deviation
SPE: solid phase extraction
TBARS: thiobarbituric acid reactive substances
TCA: trichloroacetic acid
UPLC: ultra pressure liquid chromatography
UV: ultraviolet

V: volts

v/w: volume to weight

WMA: water management area

ε: molar extinction coefficient

1. INTRODUCTION

In 2005, Cox *et al*. published an article in which they presented data indicating that most, if not all cyanobacteria contain β-*N*-methylamino-L-alanine (BMAA). This unusual amino acid had been shown previously to be neurotoxic and was hypothesized to be the causative or contributing agent to the Amyotrophic Lateral Sclerosis/Parkinsonism dementia Complex (ALS/PDC) observed amongst the Chamorro people of Guam, where it was called Lytico-Bodig disease. The hypothesis was based on the presence of BMAA in cycad seed flour and fruit bats, both of which were consumed by the Chamorro population, and on the presence of BMAA in the brain tissue of all patients succumbing to Lytico-Bodig disease. The source of the BMAA was later determined to be the symbiotic cyanobacteria in the corraloid roots of the cycads. Subsequently, BMAA was found in the brain tissue of Canadian ALS patients that could not have been exposed to BMAA via cycad seed flour or the consumption of fruit bats. This fact, combined with the report that all cyanobacteria, including free-living freshwater cyanobacteria, could contain BMAA, posed questions regarding human exposure to this toxin. Specifically the questions that needed immediate attention were whether humans could be exposed via drinking water, via ingestion of any aquatic organisms (subject to bioaccumulation of the toxin by those organisms), and whether BMAA could move through any other food web, posing a risk from consumption of higher trophic level organisms. Additionally, the potential for accumulation of the toxin in irrigated crops was of interest. Coupled to these questions were the questions of ecological significance and environmental fate of the BMAA. Specifically, was there an effect on exposed organisms and were they capable of biotransformation of the toxin or only accumulation? The formal aims were therefore:

- To determine whether BMAA bioaccumulates in cyanobacterial grazers and other aquatic organisms
- To determine whether bioaccumulated or unincorporated BMAA can be bioaccumulated by predators of cyanobacterial grazers
- To monitor toxicological effects of BMAA on all these organisms in order to evaluate their use as biosensors.
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2 LITERATURE SURVEY

2.1. BMAA

BMAA is a non-canonical, neurotoxic amino acid, with a secondary amine in the side chain (Figure 2.1a). It lacks classical structural characteristics for excitatory amino acid receptor activation. However, in its carbamate form it appears to destroy neurons via an excitotoxic mechanism (Allen et al., 1995). BMAA is polar (Jonasson et al., 2008) and was therefore expected to be readily excreted from the human body after ingestion (Strong, 2003). It is a neutral amino acid at physiological pH (Nunn and O'Brien, 1989). BMAA is capable of forming two carbamate adducts in the presence of bicarbonate namely the α-*N*-carboxy (Figure 2.1c) and the β-*N*-carboxy adduct (Figure 2.1d).

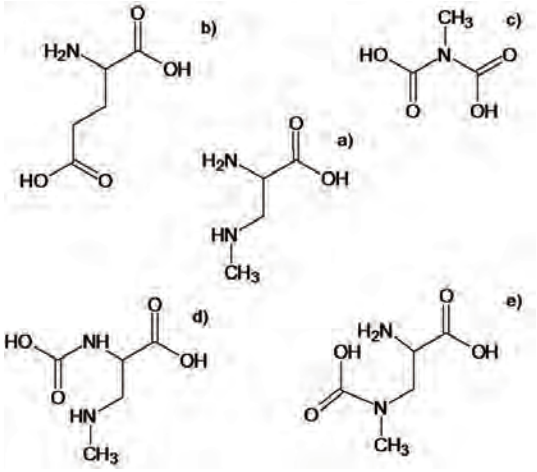

Figure 2.1: Chemical structure of a) β-*N*-methylamino-L-alanine (BMAA, 2-amino-3-methylamino-propanoic acid or L-α-amino-β-methylaminopropionic acid), b) glutamate, c) N-methyl-D-aspartate (NMDA), d) BMAA α-carbamate and e) β-carbamate.

2.1.1 Proposed neurotoxic mechanisms of BMAA

Several neurotoxic mechanisms of action of BMAA have been proposed. One such mechanism suggests that BMAA acts as a ligand to direct essential trace elements such as zinc and copper thereby leading to capture and release of metal ions (Cu^{2+}, Zn^{2+}, Al^{3+}, and Ca^{2+}) by protein-associated BMAA (Hursthouse et al., 1990). This in turn is suggested to interfere with the functioning of *N*-methyl-D-aspartate (NMDA) (Nunn et al., 1989) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Weiss et al., 1989; Carriedo et al., 1996) receptors. Alternatively, protein-associated BMAA may form dimers that chelate metal ions resulting in ionic imbalance in neurons. It has also been proposed that BMAA may have an excitotoxic effect as a glutamate agonist. BMAA competition with cystine at the Xc^- system that may result in oxidative stress (Lobner et al., 2007; Liu et al., 2009; Lobner, 2009) is also a plausible neurotoxic mechanism. BMAA protein incorporation causing: premature neural protein truncation, interference with the tertiary structure of neural proteins altering

biological activity and misfolding was also suggested as possible toxicological mechanisms. The accumulation of these biologically inactive proteins may lead to neuronal death (Murch et al., 2004a).

Irrespective of the mechanism of action, if BMAA is accumulated and released over a period of time from a protein-associated pool, chronic BMAA exposure and any one or more than one of these proposed mechanisms will lead to neural death (Murch et al., 2004a; Stipa et al., 2006).

2.1.2 In vitro BMAA neurotoxicity

Glutamate (Glu) (Figure 2.1b; Figure 2.2) is a neurotransmitter in the brain. In theory, the BMAA β-*N*-carbamate, which is isosteric to, and acts as Glu (Weiss and Choi, 1988; Weiss et al., 1989; Brownson et al., 2002) and binds to the Glu receptors in neuronal cells causing hyperactivation. This in turn leads to an influx of calcium ions into the cell causing mitochondrial damage, reactive oxygen species (ROS) generation and Glu/Ca^{2+} mediated increases in apoptotic transcription factors, sequentially activating a cascade that leads to neuronal death (Choi, 1988).

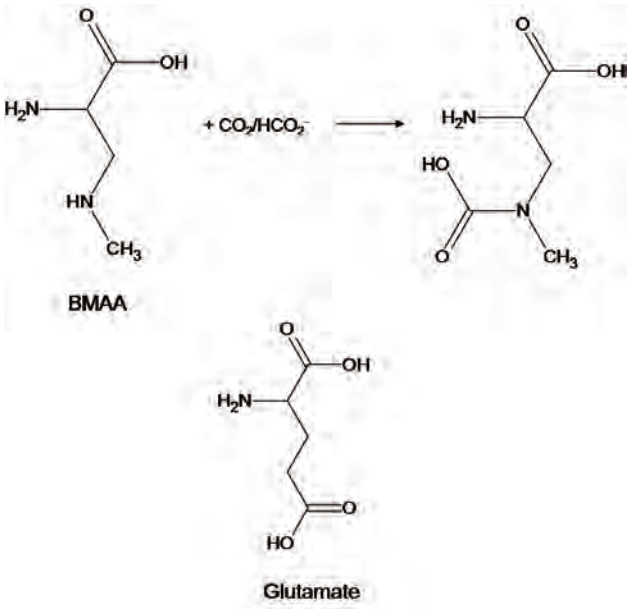

Figure 2.2: The formation of the BMAA-β-carbamate. The BMAA-carbamate has a similar structure to glutamate and can therefore mimic the action of glutamate, and cause hyperactivation of glutamate receptors (Vyas and Weiss, 2009).

Glu acts on two broad classes of ionotropic receptors, the NMDA and the AMPA/kainate receptors. *In vitro* studies show that when glutamate receptors are continuously over-

activated, neuronal injury occurs resulting in neural death (Doble, 1999; Rao et al., 2006; Lipton, 2004).

Bicarbonate-dependent BMAA neurotoxicity is possibly mediated by the activation of NMDA receptors (Ross et al., 1987; Nunn and O'Brien, 1989; Allen et al., 1995; Lobner et al., 2007). BMAA was a weak agonist of NMDA receptors *in vitro* but produced NMDA receptor-dependant neurotoxicity in cell cultures (Weiss et al., 1989). At millimolar exposure concentrations, acute neural swelling and substantial late neuronal degradation has been observed (Weiss and Choi, 1988). At lower concentrations (100 µM or below), BMAA activates NMDA receptors. NMDA receptor hyperactivation is hypothesised as the main mechanism of BMAA toxicity to motor neurons (Lobner, 2009). NMDA receptor antagonists however do not offer complete protection against the effect of BMAA, suggesting additional toxic effects of BMAA (Weiss et al., 1988; Weis et al., 1989; Lobner et al., 2007).

Since AMPA receptor antagonists, but not NMDA receptor antagonists, block the effect of BMAA *in vivo*, BMAA toxicity was considered to be mediated by AMPA/kainate receptors (Weiss et al., 1988, Weis et al*.,* 1989; Smith and Meldrum, 1990). *In vitro* studies demonstrated that BMAA acting on kainate receptors at concentrations as low as 30 µM caused an increase in intracellular calcium concentration, excess ROS production and selective motor neuron death (Rao et al., 2006) suggesting an oxidative mechanism of injury.

BMAA is an agonist of both group 1 and group 2 metabotropic glutamate receptors (mGluRs). Group 1 receptors induce phosphatidylinositol hydrolysis with inositol trisphosphate formation (Tu et al., 1998). Activation of the group 2 mGluRs cause decreased cAMP (cyclic adenosine 5'-mono-phosphate) levels that may act to protect neurons (Buisson and Choi, 1995; Genazzani et al., 1993). Cortical neuronal death has been induced by the activation of group 1 mGluRs (Strasser et al., 1998).

BMAA can cause selective motor neuron death and induce oxidative stress *in vitro* at concentrations as low as 10 µM in the presence of other neurotoxic compounds (Lobner et al., 2007; Liu et al., 2009). It is hypothesised that BMAA transport into cells occurs via the cysteine/glutamate antiporter (Xc^- system) (Lobner et al., 2007; Liu et al., 2009; Lobner, 2009). Oxidative stress would then be induced by the competition of BMAA with cysteine at the cysteine/glutamate anti-porter leading to decreased cysteine uptake, glutamate release and subsequent intercellular glutathione depletion (Figure 2.3) (Lobner et al., 2007, Liu et al., 2009). Glutathione depletion plays a role in Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Bains and Shaw, 1997; Zeevalk et al., 2000).

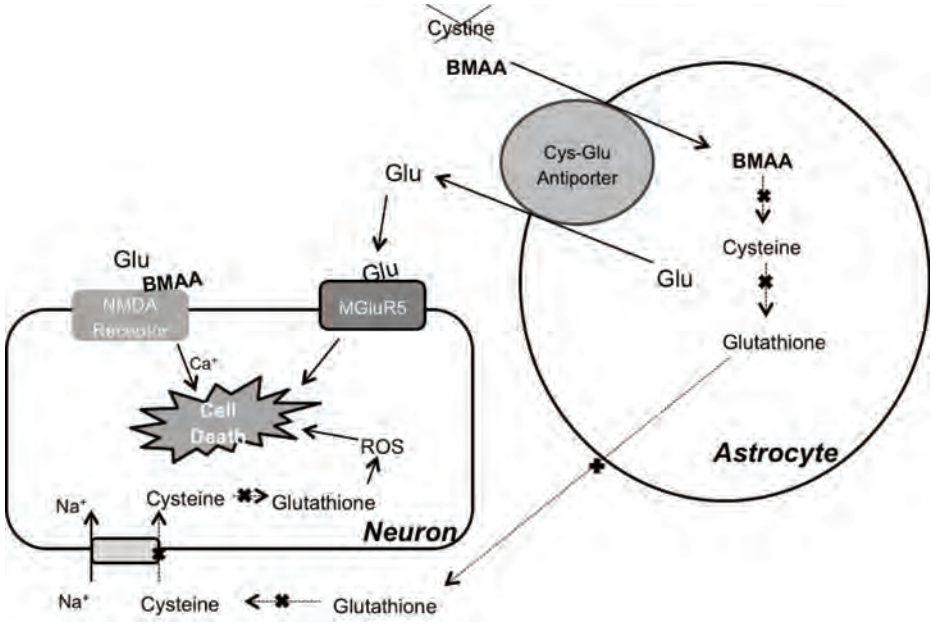

Figure 2.3: Proposed model depicting BMAA neurotoxicity (Lobner, 2009). The model is based on data obtained from mixed neural and glial cortical cultures. BMAA stimulates the release of glutamate from the astrocyte, thereby inhibiting cystine uptake. This leads to decreased astrocytic glutathione and subsequent reduced release of glutathione. This in turn limits cysteine availability for uptake by neurons. Cysteine is an essential precursor for the antioxidant glutathione in neurons. Increased glutamate released from the astrocytes cause hyperactivation of glutamate receptors, possibly leading to neuronal death (redrawn from Lobner, 2009).

To determine whether BMAA causes or contributes to human neurodegenerative disease, the toxicological effects, and mechanism of action of BMAA need to be elucidated. This information could potentially lead to therapeutic approaches (Lobner, 2009). Currently BMAA toxicology is investigated in cell culture, allowing only the investigation of the effects of acute BMAA exposure. With the theory of slow release of BMAA from a protein reservoir, chronic studies are required for full insight into the mechanism of action. Thus far, chronic BMAA exposures are limited to a small number of studies *in vivo* as described below.

2.1.2.1 In vivo BMAA toxicity

BMAA toxicity has been studied in several vertebrate models including mice, rats and primates (Karamyan and Speth, 2008). Bell et al. (1967) (as sited in Nunn, 2009) investigated the effect of synthesised BMAA stereoisomers on one-day-old domestic chicks and confirmed that only the natural L-isomer was neurotoxic to the exposed chicks. Polsky et al. (1972) found that BMAA administered to animals in high doses caused chronic neurotoxicity yet no chronic toxicity was observed with exposure to lower doses over prolonged periods. Spencer and colleagues investigated the effect of BMAA on primates and found that monkeys displayed symptoms similar to those experienced by patients in Guam after one month's exposure to BMAA (Spencer et al., 1987a; Spencer, 1987). Subcutaneous BMAA

administration to neonatal rats caused motor function and spinal cord neurochemistry changes (Dawson et al., 1998). However, Cruz-Aguado et al. (2006) could not demonstrate neurodegeneration in the presence of BMAA in rodents. Bidigare et al. (2009) suggested that BMAA from epiphytic cyanobacteria in wetlands caused a wildfowl brain disease called avian vascular myelinopathy that produced uncoordinated behaviour in affected birds. No correlation can be drawn from *in vivo* BMAA animal exposure results published to date. However, different animal models, exposure concentrations, and exposure periods were used.

Limited *in vivo* BMAA toxicological studies have been published on invertebrates. *Drosophila melanogaster* (fruit fly), *Danio rerio* (zebrafish), *Artemia salina* (brine shrimp), the protozoan *Nassula sorex* and *Daphnia magna* have been studied as model organisms. Purdie et al. (2009a and 2009b) was the first to publish BMAA toxicological studies in aquatic organisms, reporting that BMAA caused convulsions and neurological abnormalities in *D. rerio*. Neurological defects and a reduced lifespan were also observed in adult fruit flies, fed on a BMAA containing diet (Zhou et al*.,* 2009). Fruit fly larvae fed only on BMAA produced delayed motor neuron deterioration in emerging adult flies. Lürling et al. (2009) demonstrated that BMAA was not lethal to *D. magna,* however decreased motility and reproduction in adult daphnia was reported. In accordance with previous studies in invertebrates, Lürling et al*.* reported behavioural deviations in *D. magna*. From these studies, it is clear that BMAA exposure has a detrimental effect on aquatic biota and may be a potential waterborne risk to human health.

2.1.2.2 Linking BMAA to ALS/PDC

2.1.2.2.1 The Cycad Hypothesis

Following World War II, a high incidence (nearly 100 times higher than elsewhere in the world) of ALS/PDC (known by the locals as lytico-bodig) was documented among the Chamorro people of Guam (Arnold et al., 1953; Kurland and Mulder, 1954). ALS/PDC is a fatal progressive neurodegenerative disease characterized by similar symptoms to AD and/or PD (Prasad and Kurland, 1997). Epidemiological studies suggested that an environmental toxin was responsible for the high ALS/PDC rate (Torres et al., 1957; Garruto et al., 1980; Garruto et al., 1981; Reed et al., 1987). Due to the unique cultural dietary practices of the Chamorro people of Guam, the traditional diet was investigated for possible toxin sources (Reed et al., 1987; Borenstein et al., 2007). These unique components of this diet consisted of flour products made from the seeds of *Cycas micronesica* K. D. Hill, formerly *Cycas circinalis* (an indigenous cycad), flying foxes and other animals that forage on these cycads (Banack and Cox, 2003; Banack et al., 2006).

In an attempt to detect α-amino-β-oxalylaminopropionic acid (BOAA), a non-proteinogenic amino acid, which triggers the paralytic disease lathyrism, BMAA was identified in

C. micronesica (Bell and Vega, 1967; Bell, 2009). Up until 2003, it was believed that BMAA was produced by the cycad and synthesized from α-acetamidoacrylic acid (Vega and Bell, 1967). The neurotoxicity of BMAA was proven *in vivo* (mice and non-human primates) by Spencer (Spencer et al., 1986; Spencer et al., 1987a and 1987b; Ross and Spencer, 1987), and BMAA became the primary suspect of ALS/PDC occurrence on Guam.

Early BMAA investigations on Guam focused on the BMAA content of the seeds of the indigenous cycad, *C. micronesica* (Whiting, 1963). The Chamorro people traditionally consumed cycad seeds in the form of dumplings and tortillas made from cycad seed flour (Banack and Cox, 2003). Aware of the toxicity of the cycad seeds, the Chamorro people subjected the flour to repeated prolonged washing (Whiting, 1963; Kurland, 1988), which removed most of the toxic cycasin (methylazoxymethanol β-D-glucoside) as well as the BMAA (Brownson et al., 2002). Subsequently, it was argued that the flour contained too little BMAA to be the causative agent for ALS/PDC on Guam and the theory was rejected by Duncan et al. (1988, 1990, 1991). Duncan and colleagues only found small amounts of BMAA in washed cycad seeds and concluded that the BMAA concentrations used in the neurotoxicity studies conducted by Spencer et al. (1986, 1987 a, 1987 b; Ross and Spencer, 1987) were over-estimated. However, what was not considered was that washing the seeds only removed free BMAA (Murch et al., 2004a). BMAA can be associated with, bound to, or may be incorporated into, proteins (Murch et al., 2004a; Cheng and Banack, 2009) as is the case with many other non-protein amino acids (Rosenthal, 1977; Neilan et al., 1999; Rogers and Shiozawa, 2008, Bell, 2009). Protein-associated BMAA has been documented in ratios of between 120:1 and 60:1 in relation to free BMAA. Therefore, the total amount of BMAA in the cycad gametophyte and thus the cycad flour was underestimated. Washed cycad seed flour showed low amounts of free BMAA but released up to 169 μg g^{-1} BMAA on acid hydrolysis (Murch et al., 2004a). Theoretically, the protein-associated BMAA fraction is released easily by acid hydrolysis in the stomach (Cheng and Banack, 2009).

In Guam, the flying fox (*Pteropus mariannus mariannus*) is considered a delicacy and consumed whole after cooking in coconut milk. Flying foxes, which feed on *C. micronesica* seeds, were investigated as a source of BMAA. Banack et al. (2006) found that BMAA accumulated both as a free and protein-associated fraction in the fruit bat tissue. No BMAA degradation was observed as a function of cooking. Cox and Sacks (2002) found that consumption of flying foxes was confined to the Chamorro population. In the 1970s, a drastic decline in ALS/PDC among the people of Guam was documented. A corresponding decrease in flying fox numbers was noted due to over-hunting (Figure 2.4) supporting a link between ALS/PDC and flying fox consumption.

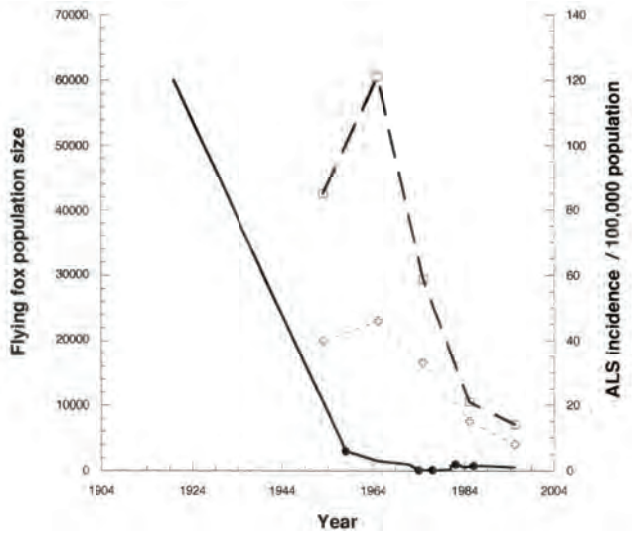

Figure 2.4. Decline of the flying fox population versus the decline in the occurrence of ALS/PDC among Guam inhabitants (Monson et al., 2003). The solid line with closed circles ( ) represents the flying fox population size. Dashed lines represents ALS incidence per 100,000 people in men (open squares) and woman (open crosses).

Other animals such as feral deer and wild pig that forage on the cycads may also supplement the BMAA input into the Chamorro people's diet (Murch et al., 2004a). In the last 50 years, ALS/PDC incidence among Guam inhabitants has decreased to approximately three times of that elsewhere in the world. This decline is paralleled with the decrease in consumption of the traditional Chamorro diet (Cox and Sacks, 2003; Cox et al., 2003; Monson et al., 2003).

Murch et al. (2004b) investigated the concentration of BMAA in blinded sets of brains of Chamorro ALS/PDC fatalities, Canadian AD fatalities and controls of tissue from Canadian donors that died of unrelated causes. All ALS/PDC brain samples contained BMAA in either or both free and protein-associated form (Table 2.1). BMAA was also detected in one of two asymptomatic Chamorro brains. Free and protein-associated BMAA was found in the brains of AD patients from Canada, whose diet did not contain any cycad product or flying foxes (Murch et al., 2004b). The mean concentration of BMAA in Canadian AD patient brain samples was 95 ± 32 µg g^{-1}, which amounts to a sixth of the mean concentration in the Guam ALS/PDC patients (Bradley and Mash, 2009). Alternative sources of BMAA input causing ALS/PDC were then considered. It was hypothesised that BMAA could be biomagnified in ecosystems other than the one described on Guam.

The findings by Murch et al. (2004b) were confirmed by that of Pablo et al. (2009) who investigated the BMAA content of the brains of AD and ALS, Huntington's disease and normal control patients that died of non-neurological causes (see Table 2.1). Similar BMAA concentrations were detected in the AD brain samples (Mash et al., 2008) compared to concentrations detected by Murch et al. (2004b). No BMAA was found in the non-neurological

disease controls or Huntington's disease brains. Mash and colleagues also investigated the occurrence of BMAA in brains from patients that died of PD and found comparable levels of BMAA to those found in AD brains (Bradley and Mash, 2009).

Table 2.1. Occurrence of BMAA in patient with ALS-PDC, AD, PD and non-neurological diseases*

Diagnosis at death	Age at death	Gender	Free BMAA (μg g^{-1})	Protein associated BMAA (μg g^{-1})
Group 1: Guam brains				
PDC	60	M	ND	1190
PDC	69	M	6.7	644
ALS	68	F	10.1	610
PDC	77	M	7	736
PDC	60	M	9.1	149
PDC	67	F	3.3	433
Asymptomatic	41	M	4.8	82
Asymptomatic	61	M	ND	ND
Group 2: Canadian non-neurological control brains				
Control				ND
Control				ND
Control				ND
Control				ND
Control				ND
Control				ND
Control				ND
Control				ND
Control				ND
Control				ND
Control				ND
Control				ND
Control				ND
Control				ND
Group 3: Canadian AD brains				
AD			3.4	220
AD			9.7	264
AD				26
AD				38
AD				53
AD				ND
AD				128
AD				34
AD				38

ND = not detected
AD = Alzheimer's disease
Asymptomatic = Guam patients who died of non-neurodegenerative causes and no clinical evidence of ALS/PC.
*Adapted from Murch et al., 2004a, 2004b; Pablo et al., 2008; Bradley and Mash, 2009

Montine et al. (2005) was however unable to replicate the findings of Murch as he could not detect the presence of BMAA in Canadian Alzheimer's or Chamorro ALS/PDC patient brain samples. Very low detection limits were reported by Montine, however only free BMAA was investigated, and these findings were not confirmed by a second analytical method. The lack of BMAA detection may be due to differences in analytical methods as Montine et al. (2005)

used standard tissue preparation methods with fluorenylmethyloxycarbonyl (FMOC) derivatization whereas Cox and Murch used the by 6-aminoquinolyl-*N*-hydrosuccinimidyl carbamate (AQC) pre-derivatization method. Based on these findings the hypothesis that BMAA is implicated in human neurodegenerative disease was rejected by Montine et al. (2005). Snyder et al. (2010) using a highly specific two-dimensional gas chromatography coupled with time-of-flight mass-spectrometry analysis followed by a targeted Parallel Factor Analysis deconvolution method, reported that no BMAA was detected in human brain samples. Similarly, several other groups have been unable to detect BMAA in complex matrices of human tissue (Rosén and Hellenäs, 2007; Kushnir and Bergquist, 2009).

Despite some contradictory evidence for the link between BMAA and ALS/PDC, there is enough supporting data to warrant further research. The BMAA-ALS/PDC hypothesis became more important with the combined evidence of BMAA biomagnification through the food chain (Cox et al., 2003), protein-association of BMAA providing a potential mechanism for slow release (Murch et al., 2004a) and the discovery of the production of BMAA by free-living cyanobacteria (Cox et al., 2005). This in turn suggests that BMAA may be of great concern to humans worldwide (Cox et al., 2005, Cruz-Aguado et al., 2006) and that further research is required to gain clarity into possible human exposure routes.

2.1.2.2.2 The Cyanobacteria Hypothesis: BMAA bioaccumulation and biomagnification

Cox et al. (2003) found that BMAA occurred in morphologically specialized positively geotropic roots of *C. micronesica*. No BMAA was present in roots with normal morphology. Cox et al. isolated symbiotic cyanobacteria of the genus *Nostoc,* which produce BMAA, from the infected coralloid roots of the *C. micronesica*.

Both free and protein-associated BMAA were detected in ascending order in the trophic levels of the traditional Guam food chain (Cox et al., 2003). Thus, the biomagnification of BMAA through the Guam ecosystem fits a classical food chain pyramid (Fig. 2.5) (Cox et al., 2003; Jonasson et al., 2008).

Nostoc, isolated from *C. micronesica*, produced 72.3 µg g^{-1} total BMAA (Cox et al., 2003). BMAA was detected at a concentration of 37 µg g^{-1} in the coralloid roots of cycads and a total of 98 µg g^{-1} in the sarcotesta of the cycad seeds. As much as 3.6 mg g^{-1} BMAA was detected in the flying foxes that forage on the sarcotesta of *Cycas micronesica* seeds (Banack and Cox, 2003; Cox et al., 2003). Therefore, an approximate 100-fold magnification was found for each trophic level (Figure 2.5). Consequently, by consuming flying foxes, the Chamorro people unknowingly consumed 10 000 times higher concentrations of BMAA than originally thought based on the analysis of the free BMAA fraction.

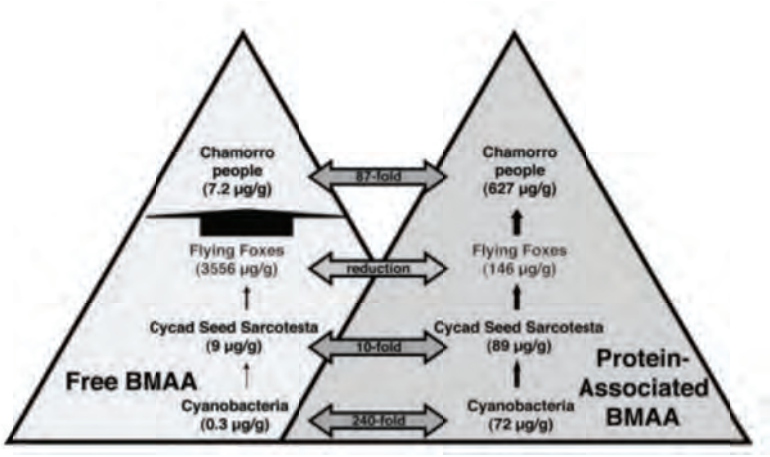

Figure 2.5. Biomagnification of BMAA through the Guam food chain (Cox et al., 2003; Murch et al., 2004a). Arrow width is proportional to the amount of free BMAA passed on to the next trophic level.

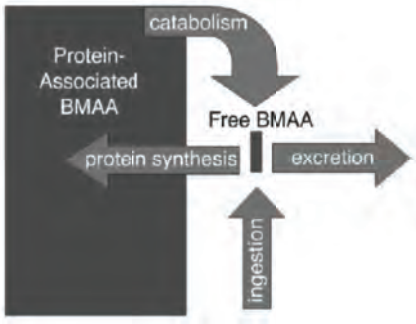

Figure 2.6. Proposed endogenous neurotoxic BMAA reservoir (Murch et al., 2004a)

Protein-associated BMAA may function as an endogenous neurotoxic reservoir (Figure 2.6) that can gradually release small amounts of free BMAA directly into the brain tissues through protein catabolism thereby causing neurological damage over a prolonged period due to chronic exposure (Murch et al., 2004a; Banack et al., 2006). This increases the potential health risk for organisms exposed to low doses of BMAA over prolonged periods and would explain the non-acute mode of toxic action (Murch et al., 2004a; Ince and Codd, 2005). Such a mechanism also explains the long latency periods observed in Chamorro people that develop ALS/PDC. It was documented that the Chamorro people who have moved away from Guam develop ALS/PDC several decades later even though they do not consume the traditional Chamorro diet any more. Similarly Filipinos (with normally low local ALS/PDC prevalence compared to Guam), who migrated to Guam developed ALS/PDC, but only many years after arrival on Guam (Torres et al., 1957; Garruto et al., 1980; Garruto et al., 1981).

Subsequently, Cox et al. (2005) analysed various strains of cyanobacteria for BMAA, and detected it in all five cyanobacterial taxonomical sections as well as 95% of all genera tested and 97% of all strains tested. It was therefore hypothesized that most, if not all, species of cyanobacteria produce BMAA (Cox et al, 2005). No correlation was observed between the free or protein-associated BMAA concentrations within or between taxonomic groups or geographic location by either Cox et al. (2005) or by Esterhuizen and Downing (2008).

The ecological function of BMAA in cyanobacteria has not been investigated (Jonasson et al., 2008). Cox et al. (2003) however suggested that BMAA in cycads may function as a chemical deterrent to herbivory due to the high concentration of BMAA found in the immature male sporangia (1,564 µg g^{-1}) and in the outer integument layer of the seed sarcotesta. Additionally, since each molecule of BMAA consists of two nitrogen atoms (Figure 2.1a), nitrogen transfer within the coralloid root/cyanobacterial symbiosis has also been suggested as a possible function (Cox et al., 2003). BMAA might serve as a nitrogen source since the plants can use amino acids as a nitrogen source (Svennerstam et al., 2007). Ionic iron is a limiting factor in cyanobacterial growth and due to the chelating abilities of BMAA; it is thought to play a role in iron binding in cyanobacteria (Wilhelm, 1995; Bradley and Mash, 2009).

Further evidence in support of the BMAA-ALS/PDC hypothesis was the detection of BMAA in the hair of Guam inhabitants that consumed the traditional diet (Banack et al., in press). BMAA was not detected in villagers who had never consumed items from the traditional Chamorro diet. As expected, disease symptoms were not associated with BMAA presence in hair. This can be explained by the long latency period before the onset of clinical symptoms in vulnerable individuals (Murch et al., 2004b). Figure 2.7 shows the accumulation of BMAA in the hair of a North American woman over a prolonged period that later developed progressive supranuclear palsy (PSP). Using hair, as a non-invasive tool, to evaluate BMAA exposure may prove useful to predict the likelihood of developing a neurological disorder.

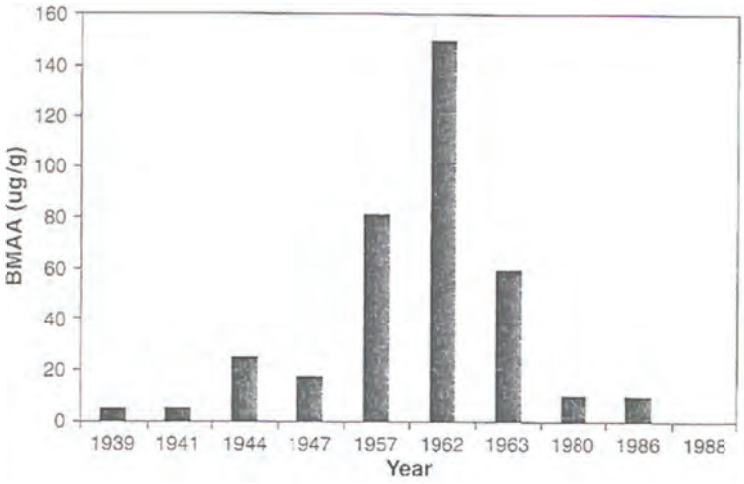

Figure 2.7. BMAA detected in hair sampled from a patient that developed progressive supranuclear palsy (PSP). No BMAA was detected from the last sample dated 1988 taken before onset of disease symptoms (Banack et al., in press)

Based on current literature, the theory of BMAA of cyanobacterial origin causing, or at least contributing to, the etiology of both endemic and sporadic ALS and AD, and possibly conferring risk for PD, is very plausible (Bradley and Mash, 2009).

2.2 Global BMAA exposure risk

2.2.1 Water consumption

BMAA has been detected in natural blooms from The Netherlands and the United Kingdom. Metcalf et al. (2008) screened 29 specimens sampled between 1990 and 2004. Free and protein-associated BMAA fractions ranging in concentration from not detected (ND) to 276 µg g^{-1} and 6 to 48 µg g^{-1} respectively were recorded. Lürling et al. (2008) tested 31 specimens sampled between 2006 and 2007, detecting both free and protein-associated BMAA ranging from ND to 27 µg g^{-1} and 3.1 to 34.1 µg g^{-1} respectively. Scum samples from an additional twenty-one water bodies were analysed and all were found to contain BMAA. Due to the presence of BMAA-containing cyanobacteria in drinking water, the human health risk via oral exposure is of great concern (Metcalf and Codd, 2009). Alternate BMAA exposure routes suggested are via spray irrigation with contaminated water and by bioaccumulation in aquatic organisms.

Tolerable daily intake and exposure guideline values for BMAA have not been established due to limited information on prevalence, incidence, and toxicology. The efficiency of standard water treatment processes to remove other cyanotoxins such as microcystin has been extensively studied but no studies on the removal of BMAA have been undertaken. In South Africa, the consecutive steps of pre-oxidation, coagulation, sand filtration, chlorination and the addition of lime are used in water treatment for the production of high quality drinking water.

Other treatment processes such as ozonation and the application of powdered activated carbon (PAC) may be employed in specific problematic situations. The efficacy of these for BMAA removal remains unknown.

The quality of water is only significant when considered in terms of its intended use or purpose (Meybeck et al., 1996). The principle aim of water treatment processes is the reliable production of water fit for domestic use from raw water sources. This must therefore result in water that is aesthetically pleasing as well as chemically safe and free of pathogens and toxins. Typical treatment processes for the production of potable water in South Africa consist of the consecutive steps of pre-oxidation, flocculation and coagulation, sand filtration, ozonation (in some cases), chlorination and a polishing lime treatment (Schutte, 2006). Pre-chlorination is typically avoided due to release of intracellular cyanotoxins and toxic by-product formation (Westrick et al., 2010) but may be applied in selected cases (Schutte, 2006).

The purpose of flocculation and sedimentation is the removal of inorganic matter and intact cells, colonies, and filaments (Pieterse and Cloot, 1997) by aggregation, using chemicals such as aluminium sulfate and ferric chloride, followed by sedimentation. In South African water treatment facilities, aluminium sulphate (between 10 and 30 mg ℓ^{-1}) and ferric chloride (between 7 and 8 mg ℓ^{-1}) are commonly used flocculants (Schutte, 2006). Complete precipitation with aluminium sulfate is a function of the pH of the water, which should be controlled between 6 and 7.4, and ferric chloride optimally precipitates at a pH of 5 to 8 (Schutte, 2006). Cyanobacterial cells, but not solubilized toxins, have been successfully removed by coagulation (Chow et al*.,* 1998; Chow et al*.,* 1999). With the removal of intact cyanobacterial cells, problematic taste and odour compounds and toxic cell metabolites are significantly reduced in the finished water. However, if an optimized chemical dose and coagulation pH was not used it may lead to cell lysis (Mouchet and Bonnélye, 1998).

Sand filtration usually follows sedimentation as a final polishing step. It is a simple procedure by which water filters through a layer of sand. There are two types of sand filtration namely rapid gravity sand filtration and slow sand filtration. Rapid sand filtration is commonly used in water treatment processes. Flow through the sand filter is achieved by gravity at a flow rate of 5-12 m hr^{-1} and the filters are cleaned by backwash at least once every day. If the washing step is performed inadequately, it will lead to cell lysis and subsequent toxin release. Low removal percentages have been reported using rapid filtration for the elimination of cyanobacterial cells and toxins (Himberg et al., 1989; Lepistö et al*.,* 1994). Slow sand filtration (0.1-0.2 m hr^{-1}) can be used as a standalone process in which case the filter is cleaned by the removal of the top layer of sand. Grützmacher et al. (2002) found that slow sand filtration was efficient in microcystin removal.

One of the most utilized disinfectants in standard water treatment is chlorine, either as chlorine dioxide gas or as chlorine compounds such as calcium hypochlorite and sodium hypochlorite. Generally, chlorination has been reported to be insufficiently effective against cyanotoxins (Hoffmann, 1976; Keijola et al., 1988; Himberg et al., 1989). The chlorination efficiency depends principally on the chloride compounds and the concentration used. Inactivation of organic compound by chlorination is pH dependant because the pKa of hypochlorous acid is 7.6 (Westrick et al., 2010).

With microcystins or nodularin, 95% removal was achieved with 1 mg ℓ^{-1} post-filtration aqueous chlorine and calcium hypochlorite, while sodium hypochlorite at the same concentration, or chloramines, only achieved 40-80% removal (Nicholson et al*.,* 1994). A chlorine residual of at least 0.5 mg ℓ^{-1} should be present after 30 min contact time in order to destroy cyclic peptides completely (Nicholson et al., 2003). Microcystin inactivation requires a pH of < 8.0 (Acero et al., 2005; Ho et al., 2006). Cylindrospermopsin, in contrast, was effectively oxidized by chlorine at pH 6-9 (Senogles et al., 2000; Nicholson et al., 1994). Application of chlorine for the inactivation of anatoxin-*a* is reportedly not suitable as this is a very slow process (Rodriguez et al., 2007). The inactivation of saxitoxins by chlorine is effective (Nicholson et al., 2003; Ho et al., 2009). The amount of organic matter present also affects the efficacy of chlorine as an oxidizing agent. Pre-chlorination of the cell with chlorine should be avoided, as it causes toxin release and produces trihalomethanes during water treatment (Tsuji et al., 1997).

PAC is widely applied in conventional water treatment processes. It is used for the removal of naturally occurring organic matter, colour (humic acid and tannins), and odour components (2-methylisoborneol and geosmin) as well as a variety of synthetic organic compounds such as pesticides. The dosage normally varies to meet the treatment needs. PAC is normally dosed in the rapid mixing area of the plant whereafter it is removed in the settling basins. Contact time may vary from 0.5 to 2 hrs or longer depending on the needs of the plant. PAC and granular activated carbon (GAC) have been shown to be effective at removing cyanotoxins (Hoffmann, 1976; Keijola et al*.,* 1988; Himberg et al*.,* 1989). PAC is, however, not efficient in intact cell removal as shown by Lepistö et al. (1994).

Ozone (O_3) is a strong oxidizing agent and is therefore frequently used in water treatment processes to remove odour compounds, to decolorize, and to control algae and other aquatic growth (Glaze et al., 1987). This very strong, broad-spectrum disinfectant is widely used and is an effective method to inactivate harmful protozoa that form cysts. It also works well against almost all other pathogens. Ozone effectively removes/inactivates microcystin (Keijola et al., 1988; Himberg et al., 1989).

Microcystins do not break down when exposed to sunlight (Tsuji et al., 1994), however ultraviolet (UV) light (between 230-245 nm) rapidly causes photolytic destruction (Tsuji et al., 1995; Senogles et al., 2000). UV as well as many additional processes can be utilized to treat specific cases. However, these processes are not very cost effective and are thus not commonly used.

Individual water treatment processes only seem to be effective for certain cyanotoxins, which is why multiple treatment processes are employed together. The aim of this study was to evaluate the efficiency of individual conventional water treatment procedures for the removal of BMAA from raw water intended for potable use so as to establish which of these treatment processes should be applied to ensure BMAA removal.

2.2.2 Food and food web based exposure

Brand (2009) reported high BMAA concentrations in fish and mollusks in the Caloosahatchee River in Florida. Similar BMAA concentrations to those found in the brain tissue of Chamorro ALS/PDC patients were reported in the brains of dolphins from the Indian River Lagoon in Florida (Mash et al., in preparation; as sited in Brand, 2009). High BMAA concentrations were reported in fish and blue crabs from Chesapeake Bay, Florida, which frequently experiences cyanobacterial blooms (Brand et al., 2010). Jonasson et al. (2010) studied BMAA bioaccumulation and biomagnification in the Baltic Sea from 2007 to 2008. Over the period of study, cyanobacterial blooms contained various amounts of BMAA. Animals feeding on cyanobacteria either directly or indirectly (by feeding on trophic levels that feed on cyanobacteria) contained BMAA. The BMAA levels increased with ascending trophic levels in agreement with the findings by Brand et al. (2010). The highest BMAA levels were detected in bottom feeding fish and filter feeders such as mussels indicating a possible human exposure route via the consumption of aquatic animals from the Baltic Sea.

Numerous dietary items tested around the world contained BMAA further pointing to human exposure risk. Roney et al. (2009) tested the delicacy *fa cai*, a Chinese soup made of the cyanobacterium *Nostoc flagelliforme*, for the presence of BMAA. Commercially available *fa cai* is partially or completely supplemented with starch noodles however traditionally available *fa cai* consisting only of *Nostoc* contained concentrations of up to 0.6 µg g^{-1} BMAA. Similarly Johnson et al. (2008) tested *llullucha*, globular colonies of *Nostoc commune* consumed traditionally in the Peru Highlands, for BMAA. BMAA was detected and confirmed in 21 Peruvian samples of *Nostoc commune* with four analytical methods. However, whether the occurrence of ALS, AD, or PD is greater among individuals who consume *fa cai* and *llullucha* was not determined. It has also been reported that several cyanobacterial dietary supplements contained large quantities of BMAA (Dietrich et al., 2008).

BMAA exposure through aerosolization has been proposed by two studies: one on the high incidence of ALS among Gulf war veterans and, the other on people residing around frequently blooming lakes in New Hampshire. An above average incidence of ALS has been reported among deployed first Persian Gulf war veterans (Kasarskis et al., 2009). It is hypothesised that cyanobacteria and cyanotoxins are carried in dust in the Gulf region (Qatar) with cyanobacterial crusts and mats widespread in this dusty and arid region. Dust is disturbed by war vehicle and soldier traffic resulting in the inhalation of BMAA, 2,4-diaminobutyric acid (DAB), and other aerosolized cyanotoxins (Cox et al., 2009). Caller et al. (2009) documented a high prevalence of ALS clusters surrounding a lake in New Hampshire that experienced sporadic cyanobacterial blooms. Hypothetically, these ALS cases may be due to chronic exposure to BMAA possibly by direct ingestion of lake water, inhalation of aerosolized BMAA, and the consumption of fish. The majority of ALS cases were documented downwind of the lake, which supports the theory of aerosolized toxins being responsible for the high incidence of ALS-PDC.

2.3 Analytical controversy

BMAA analysis remains controversial. BMAA was initially identified and quantified in cyanobacteria with pre-column derivatization by AQC followed by fluorescent high performance liquid chromatography (HPLC). The derivatizing agent reacts with primary and secondary amines leading to the formation of fluorescent derivates (Murch et al., 2004a and 2004b; Cox et al., 2005; Banack et al., 2006). Prior to the work presented in this thesis, at least five analytical techniques have been used with pre-column derivatization including HPLC with fluorescent detection (HPLC-FD), ultra performance liquid chromatography with ultraviolet detection (UPLC-UV), liquid chromatography mass spectrometry (LC-MS), and LC-MS/MS (Cox et al., 2003; Cox et al., 2005; Banack et al., 2007; Metcalf et al., 2008; Johnson et al., 2008) to verify BMAA identification. An amino acid analyser with post-column derivatization has been used as a confirmatory method (Banack et al., 2007).

BMAA was not detected in cyanobacteria by some researchers using underivatized LC-MS/MS analytical methods (Kubo et al., 2008; Rosén and Hellenäs, 2008; Krüger et al., 2010). Subsequently, concerns about misidentification, or that a compound similar to BMAA may be created during the pre-derivatization procedure were published (Rosén and Hellenäs, 2008; Krüger et al., 2010). Rosén and Hellenäs, (2008) did however detect DAB a structural isomer of BMAA. Faassen et al. (2009) and Spáčil et al. (2010) detected BMAA in cyanobacterial samples using chromatographic methods not requiring derivatization. Additionally, Banack et al. (2010) subsequently published chromatographic separation of DAB and BMAA as well as the other standard amino acids and several structural isomers of BMAA using pre-column derivatization. Recently, Papageorgiou et al. (2009) reported the absence of BMAA in cyanobacterial strains from Australia using an AQC BMAA derivatization and fluorescent detection method adapted from Cox et al. (2005). The failure of some workers to

detect BMAA in cyanobacteria, coupled with the increasing reports of enhanced sensitivity of detection of prepared BMAA standards, raised questions regarding the origin of BMAA as measured in cultures and natural samples.

2.4 Cyanobacterial Origin of BMAA

Cyanobacteria are photosynthetic prokaryotes that may produce a variety of toxic compounds including neurotoxins, hepatotoxins, cytotoxins, and lipopolysaccharide endotoxins. BMAA, an unusual amino acid first isolated from *Cycas circinalis* (Vega and Bell, 1967), is a slow acting neurotoxin that elicits neurotoxicity via various mechanisms (Rao et al., 2006; Lobner et al., 2007; Liu et al., 2009) and has been implicated in the neurodegenerative disease Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex (ALS/PDC). In 2005, Cox et al. detected BMAA within cyanobacterial cells, in 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 (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).

Despite the many reports of BMAA in cyanobacterial bloom and culture material, some investigators have been 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 the possible misidentification of the compound during analysis. However, robust method development has produced methods that clearly and distinctly distinguish BMAA from known biologically valid isomers (Banack et al., 2010, Banack et al., 2011). 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 is still lacking which hampers investment in toxicological research and epidemiology.

Some of the work described in this report deals with the confirmation of the cyanobacterial origin of BMAA, a critical prerequisite to understanding environmental fate and ecotoxicology of BMAA, and potential human exposure risk. These crucial foundation elements upon which the BMAA-ALS/PDC hypothesis stands, cyanobacterial origin, environmental fate (including food web aspects) and potential risk for human exposure, have yet to be demonstrated for freshwater systems. The aims of this work were therefore specifically to unequivocally confirm the cyanobacterial origin of BMAA, to determine whether it could enter the food web, to determine the effects of the toxin on selected model components of the aquatic ecosystem

and to evaluate the potential for bioaccumulation and biotransformation in these components. Together, this information would be used to establish whether there was any risk of exposure to humans from raw and treated water or via ingestion of any food items from the BMAA containing ecosystem.

3 MATERIALS AND METHODS

3.1 Cyanobacterial cultures and culture media

Cyanobacterial culture methods were as described by Downing et al. (2011, 2012). Briefly, cultures of axenic *Microcystis* PCC7806 and *Synechocystis* PCC6803 strains and a uni-algal strain, Synechocystis J341 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 mmol m^{-2} s^{-1} (Triton Dayglo®, Cleveland, Ohio). Culture purity was regularly monitored microscopically and by heterotrophic culture.

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 labeled ammonium ($^{15}NH_3^+$) as described by Downing et al. (2011). A brief description of the method detailed in Downing et al. (2011) is given below.

3.2.1 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 (5000 g for 10 min) and resuspended 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 hr, 4 hr and 24 hr.

3.2.2 Production not associated with primary amino acid synthesis

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

3.3 Bacterial, Plant and animal BMAA exposures

In order to establish both the uptake by and potential toxicity to various model organisms, these organisms were exposed to BMAA under controlled conditions and the uptake and, where relevant, distribution and accumulation of the toxin within the organisms determined.

3.3.1 Cyanobacteria

Mid-log cultures of *Synechocystis* PCC6803 in BG_{11} or BG_{11}^0 medium (Ripka, 1979) were exposed to exogenous BMAA (Sigma, St. Louis, MO, USA) at concentrations of 0.05 µM, 0.5 µM and 5.0 µM over 96 hours as described by Downing et al. (2012). For BMAA isotope exposure, the exposure time was 24 hr unless otherwise stated.

3.3.2 Daphnia

Daphnia magna was cultured in 20 ℓ tanks without agitation in a synthetic media as described by Wiegand et al. (2002) for several weeks prior to exposure. 50% of the medium was exchanged every 3-5 days and *Daphnia magna* were fed with living monoculture of *Scenedesmus* sp. cultivated as described by Kotai (1972). *Synechocystis* strain J341 (Esterhuizen and Downing 2008) was cultivated according to Esterhuizen and Downing (2008) but at a constant temperature of 20 ± 1°C and a photoperiod of 14:10 hours in BG_{11} media (Rippka, 1988).

BMAA exposure was performed using 100 adult daphnia in 100 ml medium with no feeding for 24 hours at 20 ± 1°C and a photoperiod of 14:10 hours. BMAA (Sigma), at nominal concentrations of 100, 500, and 1000 µg ℓ^{-1} in sterile daphnia media, was quantified by LC-MS. Controls consisting of non-exposed daphnia and BMAA in medium without daphnia were used in replicates of four. Exposed *D. magna* were removed from the exposure media after 24 hrs, washed 3 times each with fresh daphnia media and snap-frozen in liquid nitrogen and stored at -80°C for later analysis. Four replicates of 100 daphnia per 100 ml were fed BMAA containing cyanobacteria *Synechocystis*, strain J341 (Esterhuizen and Downing, 2008) at 57 million live cyanobacteria cells per day for 2 weeks. *D. magna* samples were harvested after two weeks, washed several times with media, snap-frozen and stored at -80°C.

3.3.3 Aquatic Macrophyte

C. demersum was selected based on its cosmopolitan nature. Culture and exposure methods were described by Esterhuizen et al. (2011). Briefly, *C. demersum* was cultured for 3 days in Provasoli medium consisting of deionized water containing $CaCl_2$ [0.2 g ℓ^{-1}], $NaHCO_3$ [0.103 g ℓ^{-1}] and sea salt [0.1 g ℓ^{-1}]. Plants were maintained at a constant temperature of 23°C and irradiance of 12 µmol m^{-2} s^{-1} with a photoperiod of 14:10 hours. Small section s of *C. demersum* sections (approximately 3 cm in length) were exposed to 0.5, 1, 5, 50 or 100 µg ℓ^{-1} BMAA over 24 hr. Replicates were incubated in individual containers and washed with water and methanol prior to snap freezing for later analysis. *C. demersum* sections were also exposed to 100 µg ℓ^{-1} BMAA over varying time periods (0.5, 1, 2, 4, 6 and 24 hr). Metabolism and bioaccumulation of environmental BMAA were investigated by exposing plants to 100 µg ℓ^{-1} exogenous isotopically labeled BMAA (BMAA-4,4,4-d_3,$^{15}N_2$) over a period of 48 hours.

3.3.4 Terrestrial Macrophytes

Nasturtium officinale and *Daucus carota* were selected because *N. officinale* leaves and *D. carota* roots are used in the human diet, and are usually grown by spray irrigation. *N. officinale* and *D. carota* seeds were purchased from STARKE AYRES LTD, South Africa. BMAA exposure stock was prepared using commercial BMAA from Sigma. Chemicult hydroponic medium (macroelements: 6.5% N, 2.7% P, 13% K, 7% Ca, 2.2% Mg, & 7.5% S

and micronutrients: 0.15% Fe, 0.024% Mn, 0.0024% B, 0.005% Zn, 0.002% Cu, and 0.001% Mo) was prepared by dissolving 2 g nutrient powder in 1 ℓ deionised, sterile water. To this, 0.8% bacteriological agar was added with heating to liquefy the agar. The medium was autoclaved and dispensed into specialized, sterile plant growth containers (100 mL in each container). For toxin-exposed seeds, a range of BMAA concentrations were used (100, 300, 500 µg ℓ^{-1}). The concentration of BMAA was set to a maximum of 500 µg ℓ^{-1} because this range of concentrations is normally detected in the environment (based on extrapolation from studies by Cox et al., 2005; Esterhuizen and Downing, 2008). BMAA was added to the growth medium before pouring. Seeds were surface- sterilized in three changes of 3% H_2O_2 for 5 min each followed by three washes of 10 min in sterile water. The seeds were left in fresh distilled water overnight to germinate. 16 seeds were aseptically transferred onto media in each growth container using sterile forceps. Seeds were evenly spaced in a container to ensure media and toxin availability to all samples. The plants were grown at a constant temperature of 24°C (± 1°C) with a constant continuous light source (20 photons m^{-2} s^{-1}). The root, stem and leaf lengths as well as fresh weight of 3 plants from each of the three containers from each group (BMAA-treated and control groups) were measured daily from day three for the 7-day run for *N. officinale*, and from day five for the 9-day run for *D. carota*. Three plants from each of the three containers from each group were daily placed into pre-weighed Eppendorf tubes, snap frozen in liquid nitrogen, and stored at -80°C for BMAA analysis.

Seedlings were washed in distilled water, methanol (98%), and again in distilled water to remove any surface associated BMAA. Plants were snap frozen in liquid nitrogen and lyophilized and weighed before homogenization in 0.1 M trichloroacetic acid (TCA) vigorous and repeated vortexing, and standing for 30 minutes at room temperature. Samples were then sonicated in a cold-water bath for 3 hours. Thereafter samples were centrifuged at room temperature for 10 min at 10,000 g in an Eppendorf MiniSpin benchtop centrifuge. The supernatant (containing free cellular BMAA) was removed and kept. The pellet contained the protein-associated BMAA was hydrolyzed to release the BMAA from the protein by resuspending it in 1 ml of 6 M hydrochloric acid (HCl) with 2% thioglycolic acid in an inert atmosphere at 110°C for 22 to 24 hours. The samples were filtered through a 0.22 µm filter (cellulose acetate from LASEC) after cooling. The pH of the samples was then adjusted to pH 2 with sodium hydroxide (6 M NaOH) before derivatization and analysis as described below.

3.3.5 Mussels

Four genera of fresh water mussels; *Anadonta cygnea, Unio pictorum*, *Dreissena polymorpha* and *Corbicula javanicus* were obtained from the Department of Ecological Impact Research and Ecotoxicology, at The Institute of Ecology of the Technical University of Berlin. *Anadonta* and *Unio*, measuring between 10 and 13 cm, are both native to Germany and are sediment living. *Dreissena*, a smaller mussel, measuring approximately 3 cm is an invasive mussel

originally from the Caspian Sea. These mussels live on hard substrates or, if suitable substrates are not available, on top of native mussels. This trait has lead to the larger native mussels, such as *Unio,* being unable to burrow into the sediment. This restriction on their normal behavior has resulted in them becoming endangered in their native freshwater systems. *Corbicula*, a small mussel measuring approximately 2 cm, native to Asia, is frequently used in ornamental aquaria.

Metabolism and bioaccumulation of environmental BMAA were investigated by exposing these mussels to 100 µg ℓ^{-1} exogenous isotopically labeled BMAA (BMAA-4,4,4-d_3,$^{15}N_2$) over a period of 48 hours. The experimental set-up included 16 replicates, (8 controls not exposed to BMAA and 8 expose to BMAA) each cultured individually for the entire duration for the experiment. Mussel culture media volumes were selected to allow equivalence in tissue-volume:exposure-medium-volume ratios. Smaller species; *Corbicula* and *Dreissena* were exposed to exogenous BMAA in a culture volume of 600 mL whereas the larger mussels; *Unio* and *Anadonta*, were exposed to BMAA in a total volume of 4 ℓ. The mussel culture medium, used in both routine maintenance and exposure experiment consisted of 0.106 g ℓ^{-1} $NaHCO_3$, 0.200 g $\ell^{-1}CaCl_2$ and 0.100 g ℓ^{-1} sea salt. Mussel cultures were aerated twice daily for approximately 1 minute. Whole mussels and culture medium were sampled at 24 and 48 hours following exposure (n=4). Mussels were dissected on ice, snap-frozen in liquid nitrogen and stored at -80°C until further processing. Mussel tissue samples were homogenized and free cellular amino acids were extracted with trichloroacetic acid. Free amino acid extracts were derivatized as described by Esterhuizen-Londt et al. (2011).

3.4 Evaluation of water treatment methods

Using jar-testing (Golob et al., 2005; Ebeling et al., 2003), BMAA (1 µg mℓ^{-1}) was exposed to varying concentrations of aluminium sulfate ($Al_2(SO_4)_3.16H_2O$) and ferric chloride ($FeCl_3$). Samples were stirred for 8 hrs followed by a sedimentation period of 6 hrs at a pH of 6.8 (± 0.4) for aluminium sulfate and pH 6.5 (± 0.5) for ferric chloride. Aluminium sulfate and ferric chloride exposure concentrations were 5, 10, 20, 50, 100 mg ℓ^{-1} and 2, 5, 10, 15, 20 mg ℓ^{-1} respectively in replicates of five. The BMAA concentration after flocculation was determined as described below.

Triplicate samples of BMAA (1 µg mℓ^{-1}) were filtered through 200 mm sand beds (sterile and used) by gravity at a rate of 6 ml per minute. Flow-through was concentrated on a previously optimised and tested Strata-XC Giga^{TM} tube (Phenomenex) (Esterhuizen-Londt and Downing, 2011). To test if BMAA was bound to the sand, 10 ml of BMAA (1 µg mℓ^{-1}) was filtered through a 20 mm used sand bed. The flow through was analysed as described for BMAA. The used sand was subjected to hydrolysis in an inert atmosphere in 6 N HCl containing 2% thioglycolic acid at 110°C for 22 hours (hrs). Thereafter the hydrolysate was filtered through a Whatman's no. 1 filter paper, neutralized, and analysed for BMAA as described.

Calcium hypochlorite (Sigma) was used as the disinfectant. BMAA (1 µg mℓ$^{-1}$) was exposed to 1, 3, and 6 mg ℓ$^{-1}$ calcium hypochlorite for a maximum period of 0.5 hr in replicates of three. Following exposure, samples were filtered with a 0.45 µm membrane filter, and the BMAA concentrations after chlorination determined.

PAC was investigated for its efficiency of removal of BMAA. BMAA (1 µg mℓ$^{-1}$) was exposed to 1, 5, and 10 mg ℓ$^{-1}$ of PAC for varying contact times (0.5, 1, and 2 hrs) in replicates of three. Following exposure, samples were filtered with a 0.45 µm membrane filter, and the BMAA quantified.

A Sterizone Buddy Ozone generator (Ozo Clear Marketing (Pty) Ltd trading) was used to produce ozone. The amount of ozone produced by the Sterizone Buddy Ozone generator in the chamber for a specific contact time (1, 5 and 10 min) was determined according to standard methods (Standard Methods for the Examination of water and wastewater, AWWA (1995), 4500 – O_3B) and 1 µg mℓ$^{-1}$ BMAA was exposed to these concentrations for the specified contact times.

3.5 Analytical methods

3.5.1 LC/MS analysis

Unless otherwise stated, free and protein-associated amino acids were extracted from tissue or cell pellets using trichloroacetic acid (TCA) protein precipitation and acid hydrolysis as described by Esterhuizen and Downing (2008) and Esterhuizen-Londt et al. (2011). 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 mL 20 mM HCl.

Samples were derivatized using propyl chloroformate using the EZ:faast® amino acid analysis kit (Phenomenex) and separated 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).

In all cases BMAA was detected and quantified as described by Esterhuizen-Londt et al. (2011). Briefly, a flow rate of 0.25 min and 1 µ ℓ$^{-1}$ sample injection volume were used. Column temperature was kept at 35°C. The mass spectrometer ESI source (positive ion mode) temperature was set at 250°C. The ion scan range was between 100 and 600 m/z. The interface voltage was set at 4.5 kV and the CDL voltage at -20 V with the heating block at 200°C. Data was analyzed using LCMS solutions Ver. 3 software. The liquid

chromatography-mass spectrometry system was validated using a range of dilutions of an authenticated BMAA standard (Sigma), negative controls as well as spiking 20 standard amino acids with the BMAA authenticated standard. BMAA was quantified off a standard curve of known quantities of BMAA (sigma) standard against an internal standard of either homoarginine or methionine-D3.

3.5.2 LC/MS/MS analysis

For the investigation of incorporation of labeled nitrogen into BMAA, samples derivatized as described above were analyzed 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 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.

For the analysis of production of BMAA by an axenic culture of cyanobacteria and the confirmation of de-novo synthesis of the molecule, the amino acids analyzed 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 labeled amino acids were based on LC 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.

Confirmation by product ions of BMAA and 2,4-diamonibutyric acid (2,4- DAB) was performed based on UPLC retention times and where either parent ion 333 m/z or MS/MS detection 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 for qualification were based on product ions $m/z^{+1\ amu}$ and $m/z^{+2\ amu}$ (*m/z* 246, 274 and *m/z* 247, 275).

For analyses of molecular isotopes of amino acids and BMAA, extracts were analysed by liquid chromatography/mass spectroscopy (LC/MS) using an Alliance 2695 UHPLC combined with a Micromass Quattro micro^{TM}, 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 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 (Merck, Darmstadt, Germany) (B) (flowrate: 0.25 mℓ.min^{-1} and column temperature: 35°C). BMAA was quantified using ESI (electron spray ionization) positive ion mode mass spectroscopy with an electron

spray ionization (ESI) ion source and desolvation temperatures of 150°C and 200°C respectively, scan range of 100-450 m/z and detector voltage of -1.45 kV. Nitrogen was used as cone gas at a flow rate of 15 ℓ.h^{-1} and as desolvation gas at 250 ℓ.h^{-1}. The collision cell energy (argon) was set to 2. Cone voltage was set at 20 V and the capillary voltage was 5 kV. For Multiple Reaction Monitoring the cone gas flow rate was increased to 30 ℓ h^{-1} and the desolvation gas to 40 ℓ h^{-1}. Source temperature was also reduced to 120°C and collision cell energy was increased to 13.

3.6 Transfer through trophic levels

Post mortem liver tissue samples from three crocodiles (*Crocodylus niloticus*) and one barbell (*Claria gariepinus*) recovered from the Olifants river in the Kruger National Park, South Africa, were obtained from Veterinary Services, Kruger National Park. Liver tissue samples were lyophilised overnight in a VirTris Benchtop Freeze drier (condenser temperature of -50˚C and a vacuum of 240 mTorr) and ground to a fine powder in liquid nitrogen before extraction and analysis as described above.

3.7 Responses to BMAA

3.7.1 Cyanobacteria

As described by Downing et al. (2012), *Synechocystis* PCC6803 was exposed to BMAA, 2-4-diaminobutyric acid (DAB) and a range of normal amino acids over a period of 192 hours. Samples were taken 10 minutes after the start of exposure and at 24 hr intervals for the duration of exposure. Growth rate was measure with flow cytometry as described by Downing et al. (2012).

3.7.2 Ceratophyllum demersum

C. demersum was exposed to BMAA and the responses evaluated as described by Esterhuizen et al. (2011). Briefly, plant sections were ground in liquid nitrogen and proteins extracted as per Pflugmacher (2002) with minor modifications. Total protein per extract was determined according to Bradford (1976). Catalase (CAT; EC 1.11.1.6) specific activity was measured photometrically according to Baudhuin et al. (1964), peroxidase (POD; EC 1.11.1) specific activity was measured according to Bergmeyer (1986). Glutathione *S*-transferases (GST; EC 2.5.1.18) were determined according to published methods of Habig et al. (1974) and glutathione reductase (GR; EC 1.6.4.2) activity was assayed photometrically as per Carlsberg and Mannervik (1985). Superoxide dismutase (SOD; EC 1.15.1.1) specific activity was measured photometrically using a commercial SOD assay (Calbiochem). Cellular ROS was determined with a Total Antioxidant Status Assay Kit (Calbiochem). Free radicals were quantified according to the method described by Romay et al. (1996).

Kinetic studies were performed on peroxidase and GST from *C. demersum* and three

concentrations of BMAA for 6 hours prior to measurement of enzyme specific activities against a range of substrate concentrations (Esterhuizen et al., 2011).

3.7.3 Terrestrial macrophytes

The control and BMAA-treated groups were grown as previously described for seven days. Protein extraction was done as per Pflugmacher and Steinberg (1997) with slight modifications. Plant samples were ground to a fine powder in liquid nitrogen and the resulting powder re-suspended in 2 x (w/v) sodium phosphate buffer (0.1 M, pH 6.5) containing 20% glycerol, 1.4 mM dithioerythritol (DTE) and 1 mM ethylenediaminetetraacetic acid (EDTA). Cell debris was removed by centrifugation and the supernatant precipitated with solid ammonium sulphate in two saturation steps, 35% and 80%, followed by centrifugation at 20,000 g for 20 minutes and at 30,000 g for 30 min, respectively. The precipitate from the second step, containing cytosolic enzymes, was resuspended in 2.5 mℓ of 20 mM sodium phosphate buffer (pH 7.0) and desalted by gel filtration on PD-10 Desalting Columns (GE Healthcare) before elution in 3.5 ml sodium phosphate buffer (20 mM, pH 7.0). Extracts were immediately snap-frozen in liquid nitrogen and stored at -80°C for enzyme activity assays.

Total protein per extract was determined according to Bradford (1976) with Bradford protein dye reagent (Sigma) measured at 595 nm with bovine serum albumin (BSA 98%, Sigma) used to construct standard curve. Catalase (CAT) activity was determined by spectrophotometrical measurement according to Baudhuin et al. (1964), using H_2O_2 as substrate. Decrease of H_2O_2 was followed at 240 nm for three minutes with 30 sec intervals in sodium phosphate buffer (50 mM, pH 7.0). Glutathione reductase (GR) activity was assayed spectrophotometrically as described by Carlsberg and Mannervik (1985) where the oxidation of NADPH can be monitored at 340 nm. In order to monitor the process of the reaction GSSG + NADPH + H ® 2 GSH + NADP, the decrease in NADPH was followed for three minutes at 30 sec intervals at 340 nm in sodium phosphate buffer (0.1 M, pH 7.5). Glutathione peroxidase (GPx) activity was determined spectrophotometrically according to Livingstone et al. (1992) using hydrogen peroxide as substrate. This assay relies on the capability of GR to generate GSH from GSSG in a NADPH dependent reaction. The rate of $NADP^+$ formation was monitored for three minutes at 30 sec intervals at 340 nm in sodium phosphate buffer (0.1 M, pH 7.5). Superoxide dismutase (SOD) was measured spectrophotometrically using a commercial SOD kit (Sigma-Aldrich™).

Enzymatic activity was expressed in in katal (kat) per milligram of protein (kat mg^{-1} prot), where 1 kat is the conversion of 1 mol of substrate per second. The enzymatic activity of CAT, GR, SOD, and GPx is calculated by $z = (\Delta E \,.V) / (\varepsilon \,.\, d \,.\, \Delta t \,.\, v \,.\, c)$, with ΔE = extinction change per minute, V = test volume (µℓ), ε = molar extinction coefficient (ℓ $mmol^{-1}.cm^{-1}$), d = cuvette width, Δt = measuring interval (time unit), v = volume of enzyme extract in the test (µℓ), c = protein content of the enzyme extract. All enzymatic measurements were performed

in triplicate for each sample, except for SOD, where four replicates were used.

3.7.4 Daphnia magna

Protein extraction was performed as per Wiegand et al. (2000). *D. magna* samples were homogenized with sodium phosphate buffer (0.1 M, pH 6.5), containing 20% glycerol, 1.4 mM dithioerythritol (DTE) and 1 mM ethylenediaminetetraacetic acid (EDTA). Cell debris was removed by centrifugation at 10,000 x G and the microsomal fraction obtained by centrifugation at 105,000 x G for 60 min. Microsomes were resuspended in 20 M sodium phosphate buffer containing 20% glycerol. Soluble proteins were concentrated by ammonium precipitation at 35% and 80% saturation followed by centrifugation at 30,000 G for 30 min. The resulting pellet was resuspended in 1 ml of 20 mM sodium-phosphate buffer (pH 7.0) and desalted by gel filtration on NAP-5 columns (Amersham Pharmacia) with elution in 1 mℓ of sodium buffer. Extracts were immediately snap-frozen and stored at -80ºC.

Total protein was determined according to Bradford (1976) with Bradford protein dye reagent (Sigma) measured at 595 nm. Superoxide dismutase (SOD) was measured photometrically using a commercial SOD assay (Calbiochem). Catalase (CAT) was measured photometrically according to Baudhuin et al. (1964) at an absorbance of 240 nm. Glutathione S-transferase (membrane associated, mGST and soluble, sGST) was measured photometrically at 340 nm according to Habig et al. (1974) using CDNB (1-chloro-2,4-dinitrobenzene) as substrate.

3.8 Production of molecular isotopes of BMAA

BMAA isotopes 119.15 amu and 123.34 amu were produced using a proprietary method. Although we were provided with the method and given permission to use it, we are not able to disclose the method used.

3.9 Metabolism of BMAA

In order to evaluate the potential for either bioaccumulation or biotransformation of environmental BMAA, molecular isotopes of BMAA were synthesized and selected model organisms were exposed to these BMAA isotopes. The redistribution of isotopic nitrogen was then monitored throughout the amino acid pool to determine whether, and potentially how, BMAA was metabolized.

3.9.1 Use of isotopic tracers

Detection and analysis of isotopically labeled amino acids were based on HPLC retention times and mass spectrometric detection of parent ion $m/z^{+x\ amu}$, where x equal 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, normalized 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 labeled or unlabelled amino acids was performed. Data presents the relative abundance of labeled amino acids based on LC/MS peak areas, unless otherwise stated.

3.10 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 identifying if significant differences existed between treatment groups (alpha=0.05). Post hoc comparisons were done using the Tukey HSD test. Statistical analysis of de novo synthesis of BMAA data is described in Downing et al. (2011).

4 RESULTS AND DISCUSSION

4.1 BMAA is a cyanobacterial metabolite

Cellular BMAA decreased within 10 min of exposure of nitrogen-deprived cells to ammonia. Exposure to nitrate had a similar but delayed effect suggesting that reduction of the nitrate to ammonia was required to result in the decrease in BMAA content (Downing et al., 2011). *Microcystis* PCC7806 grown with $^{15}NH_4^+$ as sole nitrogen source, incorporated ^{15}N into free amino acids and proteins within 24 h of exposure. Exposure of nitrogen deprived *Microcystis* PCC7806 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, within ten minutes after exposure to the heavy combined nitrogen. The observed increase 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).

However, no increase in ^{15}N-labelled BMAA was detected over the same period suggesting that BMAA was not made via normal amino acid biosynthetic reactions. This deduction is supported by the absence of any increase in ^{15}N labeled sarcosine, a molecule that is similarly not synthesized during primary amino acid synthesis. Subsequent 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, in 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 and physiological roles for BMAA in cyanobacteria.

4.2 BMAA bioaccumulation

The accumulation of BMAA by a range of aquatic organisms is of importance not only from a human exposure risk assessment perspective but also because of potential ecotoxicity and the possibility of biotransformation and consequent application in bioremediation.

4.2.1 Cyanobacteria

As described by Downing et al., (2012), *Synechocystis* PCC6803 showed a concentration dependent uptake of exogenous BMAA but no significant difference in the protein-bound cellular BMAA fraction over 96 hours. The apparent decreasing trend in protein associated BMAA was suggestive of a non-primary protein association because of the rapid association and subsequent decrease at a rate not comparable with protein turnover. Uptake appeared saturable but whether this in indicative of a maximum cellular concentration or equilibrium with a biotransformation process remains to be determined. Since cyanobacteria are able to synthesize BMAA it is conceivable that they are able to maintain cellular levels at appropriate

physiological concentrations. However, biotransformation by a producing organism is of little academic interest and of no practical use in bioremediation.

4.2.2 Daphnia

A 6 ± 0.5% (mean ± SE) loss was seen in the control, over a period of 24 hours. BMAA was taken up by *D. magna* in a dose dependent manner and internalized free BMAA increased with increase in BMAA exposure concentration (Figure 4.1). Taking the average loss of BMAA not due to uptake into account, the disappearance of BMAA from exposure media correlated with appearance in cells with only minor amounts not accounted for. No protein-associated BMAA was detected in *D. magna* for all exposure concentrations after a 24 hour exposure.

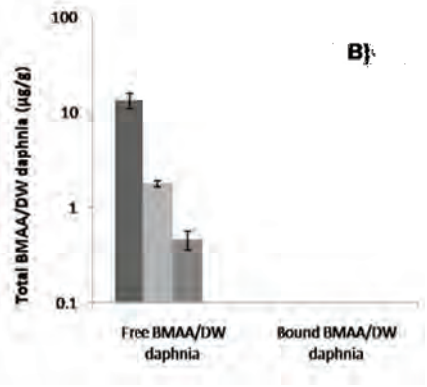

Figure 4.1. BMAA content of *D. magna* after an exposure period of 24 hours to an authenticated BMAA standard (Sigma) of varying concentrations. Error bars denote standard deviation of n = 4. ■ BMAA exposure concentration of 1000 µg ℓ^{-1}, ■ BMAA exposure concentration of 500 µg ℓ^{-1}, ■ BMAA exposure concentration of 100 µg ℓ^{-1}, ■ Negative control with no added BMAA. (Esterhuizen-Londt, PhD Thesis, 2010. Article in preparation for submission for publication).

Figure 4.2 shows BMAA clearance from the exposure media and its uptake into the *D. magna*. The daphnia cleared BMAA from the media at an average rate of 2.618 µg hr^{-1} over 24 hours. Free BMAA within *D. magna* appeared at an average rate of 0.011 µg hr^{-1} over 24 hours. No protein-associated BMAA could be detected in the organisms.

Synechocystis strain J341 (Esterhuizen and Downing, 2008) contained 0.571 µg g^{-1} free and 2.511 µg g^{-1} protein-associated BMAA under the growth conditions used. After feeding *D. magna* the approximately 57 million cyanobacteria cells per day for 2 weeks no BMAA could be quantified in free or protein-associated fractions in *D. magna*. The short time exposure to BMAA resulted in its uptake by *D. magna* but no protein-association.

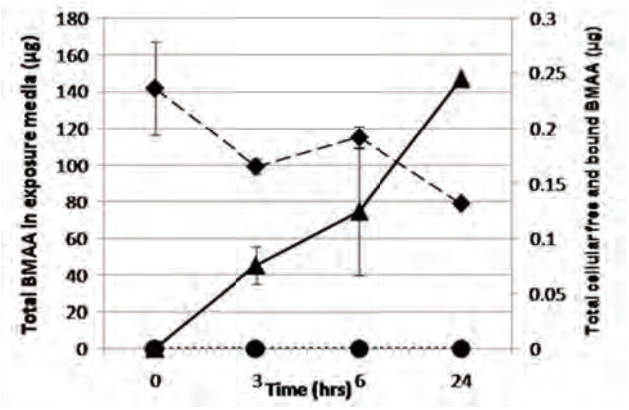

Figure 4.2. BMAA clearance from exposure media of 1000 µg L^{-1} BMAA and appearance of free and protein-associated BMAA in *D. magna* over a period of 24 hours. Error bars denote standard deviation of n = 4. –◆– exogenous BMAA in exposure media; —▲— total cellular free BMAA in taken up by daphnia; ···●·· protein associated cellular BMAA in daphnia. (Esterhuizen-Londt, PhD Thesis, 2010. Article in preparation for submission for publication).

Approximately 0.2% of the initial BMAA in the exposure media was taken in by the daphnia. Unlike *C. demersum* (Esterhuizen et al., 2010), *D. magna* contained no detectable protein-associated BMAA. Reasons for this might include the capability of plants to absorb amino acids from the surrounding media, different cellular protein turnover time, or BMAA binding affinity for plant proteins. However, the uptake of free BMAA by *D. magna* still offers opportunity for its transfer to predators of *D. magna*. A further support of free BMAA uptake into aquatic animals was evidenced by malformations during zebrafish embryo development caused by BMAA exposure (Purdie et al., 2009). Lürling et al. (2010) found very high bioconcentration factors for BMAA exposed daphnia, however, the exposure period to BMAA was up to 15 times longer and the experiment was performed with juvenile and adult daphnids whereas the experiment reported here was limited to adult daphnia. Lürling et al. (2010) did not, however, distinguish between free and protein-associated BMAA.

No BMAA was detected in daphnia cultured solely on BMAA-containing *Synechocystis*. Rohrlack et al. (1999) found daphnia fed on *Microcystis* had a reduced ingestion rate. Even though the cyanobacterial cell size of *Synechocystis* is suitable for daphnia consumption, cells might have passed through a daphnids gut undigested as it also happens for other cyanobacteria (Fulton and Paerl, 1987). Morphological features, such as size or mucus, might hamper ingestion or digestion of cyanobacteria by daphnia (Fulton and Paerl, 1987; De-Benardi and Giussani, 1990) resulting in insufficient release of BMAA from the cyanobacterial cells.

4.2.3 Aquatic Macrophyte

As described by Esterhuizen et al. (2010), significant amounts of BMAA were taken up by *C. demersum* at tested exposure concentrations. The total BMAA in the plant section at 24 h was linearly related to the exposure concentrations suggesting saturable uptake at a

concentration below 100 µg ℓ^{-1}. Between 3% and 13% of the total BMAA in the plant sections was protein associated. Furthermore, BMAA removal from the media corresponded to the appearance of BMAA in both free and bound forms in the plant sections but although BMAA was clearly taken up by *C. demersum*, bioaccumulation in a form that may result in exposure to consuming organisms cannot be confirmed without evaluating the potential for biotransformation or loss of BMAA to BMAA-free medium. These issues are addressed later in this report.

4.2.4 Terrestrial Macrophytes

Both free and protein-associated BMAA fractions in exposed plants increased in accordance with exposure concentrations.

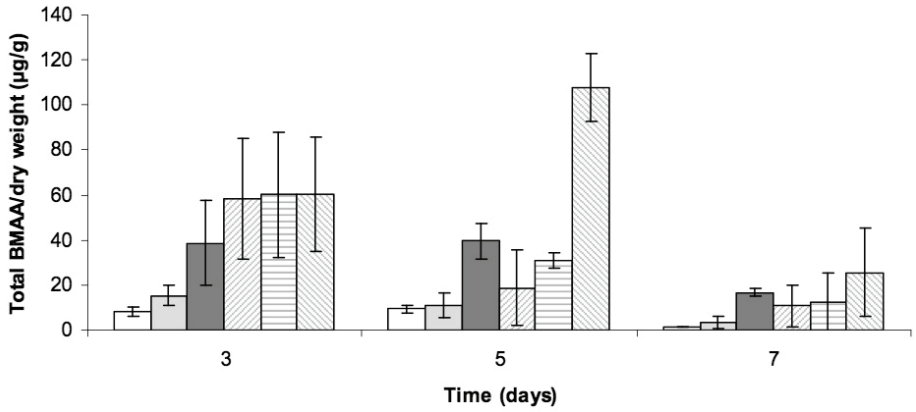

Figure 4.3. BMAA uptake by *N. officinale* expressed per dry weight of the sample after 3, 5, and 7 days at various BMAA concentrations: total cellular free BMAA extracted from *N. officinale* when the exposure concentration was 100 (□), 300 (■), and 500 µℓ^{-1}(■); total cellular protein-associated BMAA obtained when the exposure concentration was 100 (diagonal left rising to right), 300 (horizontal lines), and 500 µℓ^{-1} (diagonal left dropping to right). Data are mean ± SD (n = 3). Control plants not exposed to BMAA had no free or bound cellular BMAA. (Niyonzima, MSc dissertation, 2010. Article in preparation for submission for publication).

Total protein associated cellular BMAA concentrations were higher than free cellular BMAA concentrations for all BMAA exposure concentrations. The total free cellular BMAA increased slightly from day 3 to day 5, and then declined from day 5 to day 7 in plants treated with 100 and 500 µg ℓ^{-1} BMAA; but it decreased from day 3 to day 7 in plants treated with 300 µg ℓ^{-1} BMAA. The total bound cellular BMAA decreased from day 3 to day 7 at 100 and 300 µg ℓ^{-1}; however, at 500 µg ℓ^{-1}, it increased from day 3 to day 5, and then decreased from day 5 to day 7 (Figure 4.3). Control plants not exposed to BMAA had no free or bound cellular BMAA.

Both free and bound cellular BMAA expressed per dry weight of the *D. carota* sample increased as the concentration of BMAA in the growth medium increased. The total bound cellular BMAA concentration was also higher than free cellular BMAA concentration for all

BMAA exposure tested (Figure 4.11). In addition, total free and bound cellular BMAA decreased from day five to day seven for all exposure concentrations. Control plants not exposed to BMAA had no free or bound cellular BMAA.

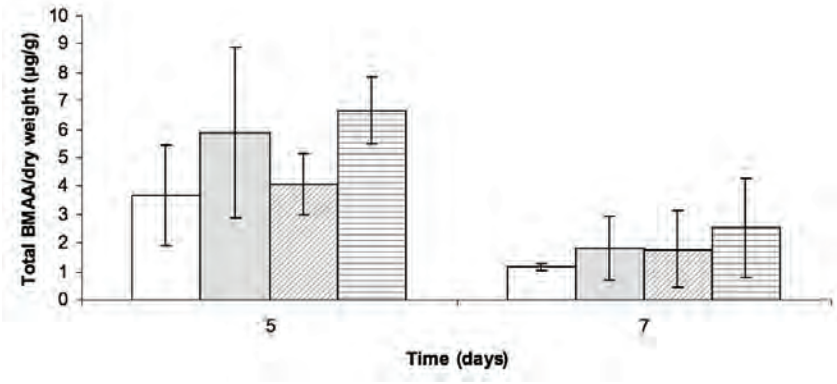

Figure 4.4. BMAA uptake by *D. carota* expressed per dry weight of the sample after 5 and 7 days at various BMAA concentrations: total cellular free BMAA extracted from *D. carota* when the exposure concentration was 100 (□) and 500 µg ℓ^{-1}(■); total cellular protein-associated BMAA obtained when the exposure concentration was 100 (diagonal lines), and 500 µg ℓ^{-1}(horizontal lines). Data are mean ± SD (n = 3). Control plants not exposed to BMAA had no free or bound cellular BMAA. (Niyonzima, MSc dissertation, 2010. Article in preparation for submission for publication).

It is interesting to note the trend indicating reduction in cellular BMAA, both bound and free, between days five and seven in both species. This may indicate catabolism or covalent modification of BMAA.

4.2.5 Mussels

Exogenous BMAA was taken up by all four freshwater mussels species; *Dreissena, Corbicula, Anadonta* and *Unio.* The uptake of exogenous BMAA was substantial with up to 90% removal of BMAA from the culture medium within 48 hours by certain species. Data in Table 4.1 show the amount of BMAA removed from the solution and the amount of free cellular BMAA in the soft tissue of respective mussel species following 24 or 48 hours of exposure to exogenous BMAA. The amount of BMAA removed from the culture medium varied significantly between mussel species but not as a function of mussel size, suggesting different filtration and uptake efficiencies. BMAA loss from the culture medium due to other non-mussel related biological activity, such as natural or bacterial degradation, was relatively small over a total of 4 days. Two mussels species, *Corbicula* and *Unio* released BMAA back into the culture media within 24 hours of being placed in BMAA-free medium following either 24 or 48 hours exposure to exogenous BMAA. *Cobricula* released up to 74 µg (±6 µg) BMAA following 24 hours exposure to BMAA and 33 µg (±13 µg) following 48 hours exposure to exogenous BMAA. *Unio* released up to 50 µg (±13 µg) and 15 µg (±10 µg) following 24 and 48 hrs exposure to exogenous BMAA, respectively. The large discrepancy between BMAA

loss from the culture medium and the actual amount of BMAA within the mussel tissue suggests that BMAA is either being metabolized or covalently modified by the mussel. The fact that BMAA is also released back into the medium by some species, with the amount of BMAA released being larger than the amount of BMAA detected in the organisms suggests that BMAA is covalently modified within the organism and that this modification is temporary and reversible, resulting in release of BMAA from some covalently modified form within the mussels.

Table 4.1. BMAA loss from solution and bioaccumulation in mussels.

Mussel	BMAA Uptake (µg lost from culture medium)				BMAA bioaccumulation (µg free BMAA in mussel soft tissue)			
	Within 24 hours		Within 48 hours		Within 24 hours		Within 48 hours	
Dreissena sp.	**12.5**	±3.2	**47.6**	±2.0	**0.3**	±0.18	**0.74**	±0.5
Corbicula sp.	**2.5**	±2.7	**21.2**	±6.0	**2.6**	±0.8	**3.0**	±0.95
Anadonta sp.	**12.5**	±3.2	**47.6**	±2.0	**0.12**	±0.01	**0.1**	±0.005
Unio sp.	**252**	±9.8	**297**	±16.0	**0.096**	±0.004	**0.09**	±0.008

If the labeled BMAA distributes the primary amino nitrogen isotope to other amino acids this would indicate catabolism. However, it is as likely that the modified form of BMAA is a protein associated form and this requires further investigation as such a form would pose a risk to consumers of these organisms.

4.3 Treatment of water

Aluminium sulfate or ferric chloride did not interact with BMAA at the typical concentrations used and BMAA was therefore not removed from the solution (Figure 4.5). There was no significant difference between treated samples compared to untreated controls ($p=0.465$ for aluminium sulphate and $p=0.3468$ for ferric chloride). As BMAA is such a small molecule with a neutral charge, interaction was not expected. Flocculation is very effective in removing cyanobacterial cells (Chow et al., 1998, 1999) and all intracellular BMAA can therefore be removed if pre-chlorination is avoided.

Rapid sand filtration was successful at removing BMAA from samples (Figure 4.6). As BMAA has the ability to bind to proteins (Murch et al., 2004), interaction with silica, or organic matter associated with the sand is plausible. BMAA removal by the sand however becomes less efficient as the sand accumulates material (Figure 4.6). The BMAA therefore presumably interacts with the sand directly, possibly via ionic interactions.

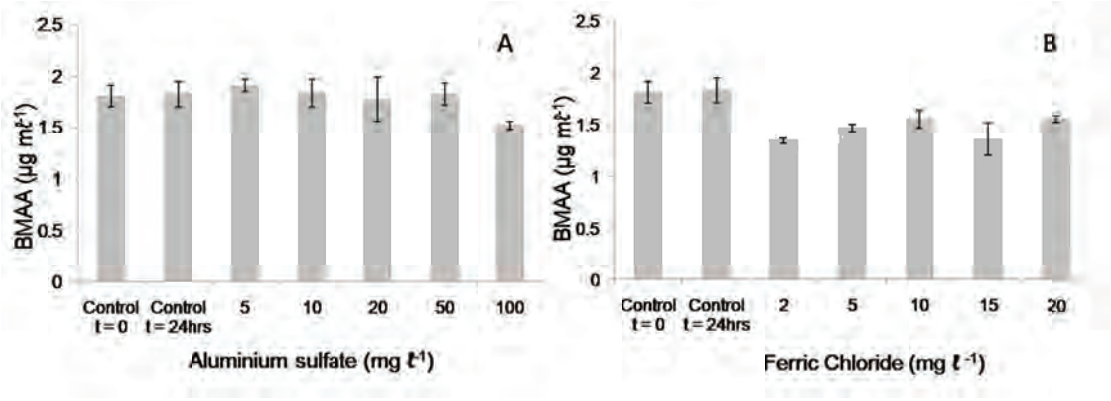

Figure 4.5. The exposure of 1 µg m ℓ^{-1} BMAA to varying concentrations of A) aluminium sulphate and B) ferric chloride. BMAA recovered by SPE concentration prior to derivatization followed by analysis by LC-MS. Data are mean ± SD (*n* = 5). Significant differences ($p < 0.05$) to an unexposed control are indicated with a *. (Esterhuizen-Londt, PhD Thesis, 2010. Article in preparation for submission for publication).

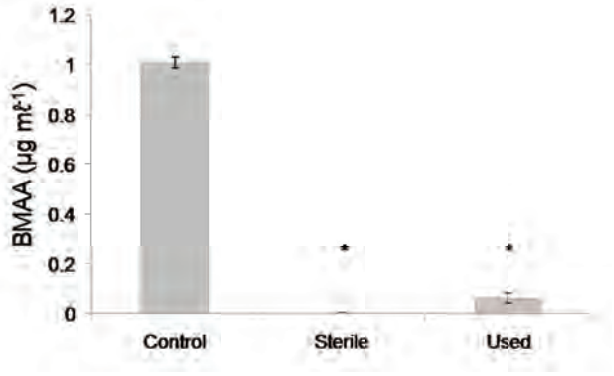

Figure 4.6. BMAA (1 µg mℓ^{-1}) passed through new and used sand by gravitational force, collected by SPE, concentrated prior to derivatization and analysis by LC-MS. The control sample was not passed through the sand filter. Data are mean ± SD (*n* = 3). Significant differences ($p < 0.05$) to an unexposed control are indicated with a *. (Esterhuizen-Londt, PhD Thesis, 2010. Article in preparation for submission for publication).

Although, sand filtration was not previously found to be effective against cyanotoxins (Hitzfeld, 2000), significant amounts of BMAA were removed by filtration through both new and used sand compared to the control. The majority of BMAA bound to the sand was recovered by hydrolysis (Figure 4.7) suggesting that the BMAA is not metabolized or altered in any way but associated directly with the filter material and/or microbiological components of the mature filter.

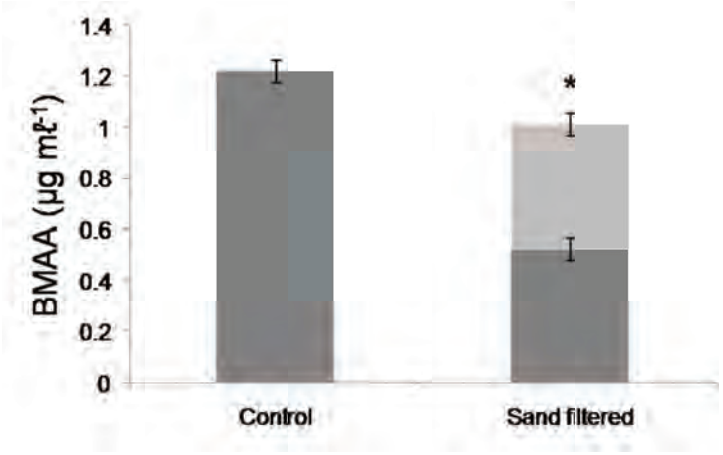

Figure 4.7. BMAA passed through used sand. Control was not passed through sand. Free BMAA (■) is the amount of BMAA in the elutant after passing through the sand filter and bound (■) is the amount of BMAA released from the sand after hydrolysis. Data are mean ± SD (*n* = 3). Significant differences ($p < 0.05$) to an unexposed control are indicated with a *. (Esterhuizen-Londt, PhD Thesis, 2010. Article in preparation for submission for publication).

Chlorine is a very potent oxidizing agent and oxidized BMAA at all tested concentrations over an exposure time of 0.5 hr (Figure 4.8). There is a significant difference ($p<0.05$) between all treated samples and the control. Using only 1 mg ℓ^{-1} calcium hypochlorite 99.9% of BMAA was removed from the sample. Higher concentrations tested removed BMAA to below the limit of analytical detection.

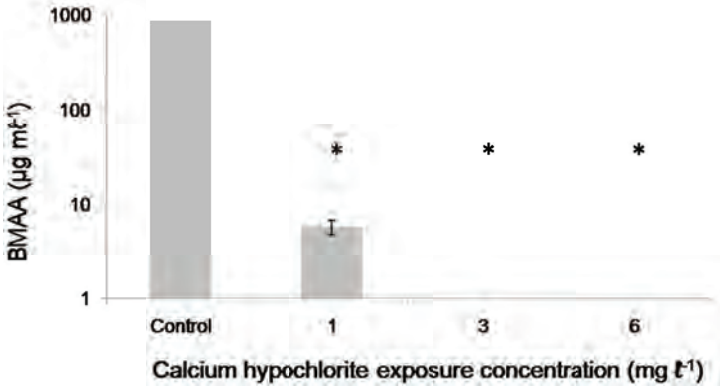

Figure 4.8. BMAA (1 µg $m\ell^{-1}$) exposed to varying concentration of calcium hypochlorite (contact time is 1 hour). Data are mean ± SD (*n* = 3). Significant differences ($p < 0.05$) to an unexposed control are indicated with a *. (Esterhuizen-Londt, PhD Thesis, 2010. Article in preparation for submission for publication).

Free BMAA was successfully removed with the application of PAC (Figure 4.9). PAC BMAA binding increased dose dependently and with an increase in contact time. At the concentration and contact time commonly used (10 mg L^{-1} and 2 hrs contact time) the majority of BMAA (99.8%) was removed.

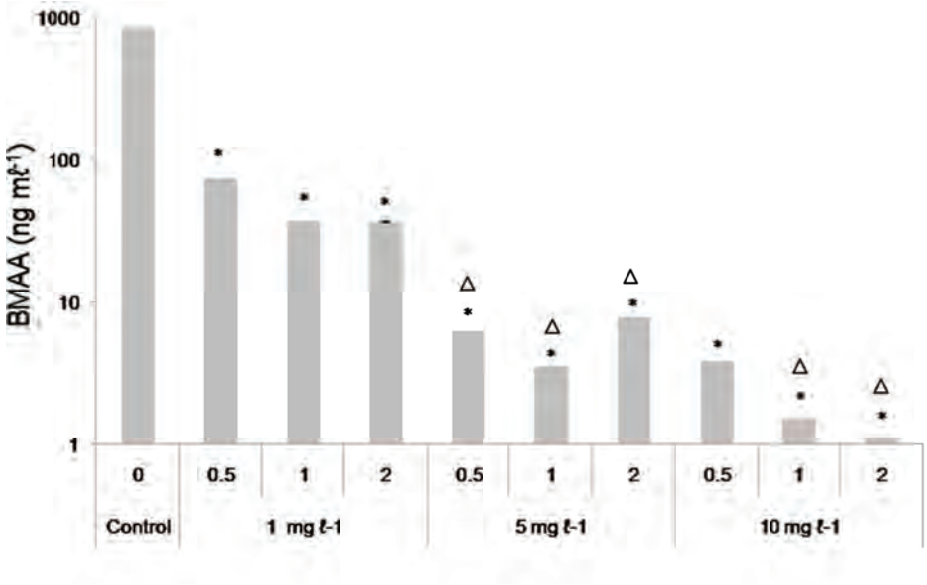

Figure 4.9. BMAA (1 µg mℓ$^{-1}$) exposed to varying concentrations of PAC for various contact times. Standard deviations are shown (*n*=3) however standard error bars cannot seen with the logged axis. Significant differences ($p < 0.05$) to an unexposed control are indicated with a *. Significant differences ($p < 0.05$) to the previous exposure concentration for the same exposure time are indicated with a Δ. (Esterhuizen-Londt, PhD Thesis, 2010. Article in preparation for submission for publication).

In this study, the maximum concentration of ozone generated by the Ozone Buddy^{TM} was 1.7 mg ℓ$^{-1}$ ozone after 5 min. Increased generation time did not increase the amount of ozone generated as ozone rapidly degrades in water. Ozone concentrations generated were therefore much lower than used in standard water treatment processes (7 mg ℓ$^{-1}$, > 4 min). When BMAA was exposed to 1.7 mg ℓ$^{-1}$ of ozone for 10 min, no oxidation of the molecule was achieved. In previous studies it was reported that ozone is more effective than chlorine in destroying microcystin-LR, anatoxin-*a* and paralytic shellfish poisons (Rositano et al., 1998). With addition of organic material higher concentrations of ozone and a longer contact time was required to remove all toxin (Rositano et al., 1998). No significant difference was observed between the ozone treated BMAA samples and the control (data not shown).

4.4 BMAA through two trophic levels

Both microcystin (data not shown) and BMAA were detected in both the catfish and crocodile samples (Figure 4.10).

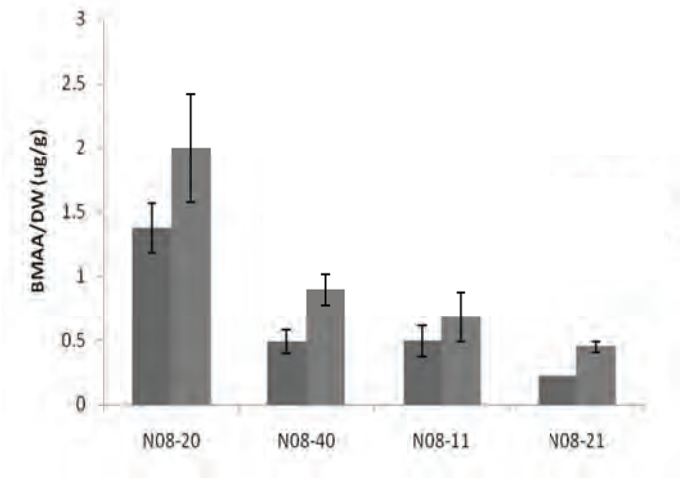

Figure 4.10. BMAA content per dry weight of liver tissue samples of N08-20 (female adult crocodile), N08-40 (male sub-adult crocodile), N08-11 (female sub-adult crocodile), and N08-21 (male adult Barbell). Free BMAA( ) and Bound BMAA ( ). Error bars denote standard deviation (*n*=3). (Article in preparation)

These data constitute the first evidence of transfer of BMAA through a freshwater food web from source cyanobacteria into catfish and subsequently into crocodiles that were observed to be gorging on the catfish over a period of time (D Govender [SANParks], pers. com.). The diet of the catfish is varied on consists of fish, amphibians and any appropriately sized carrion. The presence of both microcystin (data not shown) and BMAA in the catfish and crocodiles suggests that the transfer of these toxins probably occurred through several additional trophic levels indicating a high level of persistence and increased potential for human exposure.

4.5 Response to BMAA

The ecotoxicology of BMAA is intimately linked to its environmental fate and the potential for human exposure to the toxin. This section deals with evaluating basic indicators of toxicity in the model organisms tested.

4.5.1 Cyanobacteria

The presence of exogenous BMAA in the growth medium resulted in a concentration dependent decrease in the growth of *Synechocystis* PCC6803 (Downing et al. 2012). Within 48 hours of exposure to the lowest tested concentration (4.2 µM) a significant ($p < 0.05$) decreases in growth were observed. Significantly ($p < 0.05$) reduced growth was still observed after 192 hours. The decrease in growth was accompanied by chlorosis and a decrease in intact cells was observed at 50 µM. At the lower exogenous BMAA concentration of 4.2 µM, growth was retarded but not inhibited. Following 192 hours of exposure to exogenous amino acids, Downing *et al*. (2012) reduced the light intensity from 16 $\mu mol.m^{-2}.sec^{-1}$ to 10 $\mu mol.m^{-2}.sec^{-1}$, which resulted in the recovery of growth and photosynthetic pigmentation in the presence of 4.2 µM and 42 µM exogenous BMAA.

The negative effect of exogenous BMAA on the growth of *Synechocystis* PCC6803 were therefore both BMAA concentration and light dependent and very similar to the effects of nutrient, and specifically nitrogen, deprivation and/or high light stress. The induction of this cellular state may therefore indicate a role for BMAA in regulation of this response, particularly since BMAA is produced under conditions of nitrogen deprivation, or may simply represent an oxidative stress response to non-physiological concentrations of the toxin that has been shown to induce oxidative stress.

4.5.2 Daphnia

BMAA inhibited the activity of the oxidative stress response enzymes of the exposed daphnia thereby possibly inducing oxidative stress due to a lack of ability to clear ROS from the cell. The SOD and CAT belong to the group of enzymes responsible for reduction of oxygen or reactive oxygen species (ROS). The activity of the tested antioxidant enzymes was significantly ($p<0.05$) lower compared to the control for all BMAA exposure concentrations (Figure 4.11).

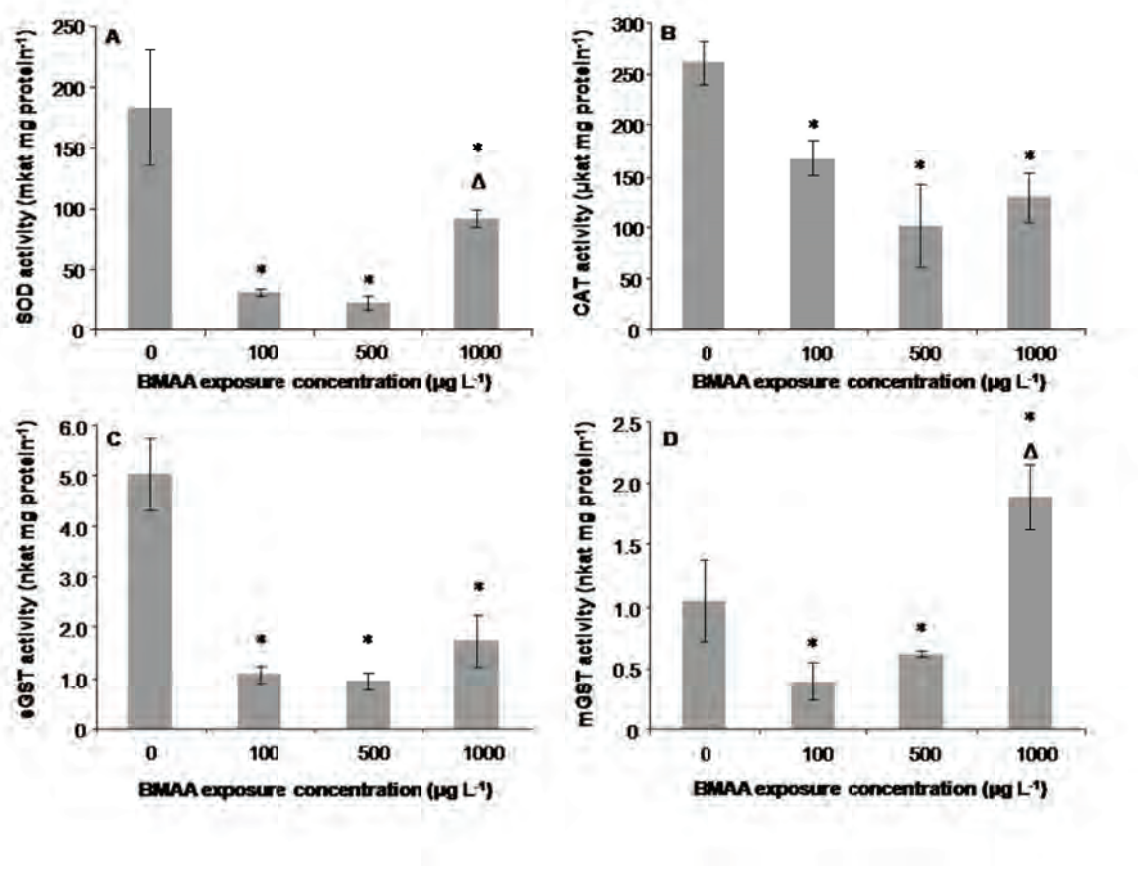

Figure 4.11. Activities of antioxidant and biotransformation enzymes in *D. magna* exposed to increasing concentrations of BMAA. A) Superoxide dismutase, B) catalase, C) cytosolic glutathione S-transferase, D) membrane bound glutathione S-transferase; (mean and standard deviation of n = 4, $p < 0.05$ * denotes difference to control, Δ denotes difference to other BMAA treatments) (Esterhuizen-Londt, PhD Thesis, 2010. Article in preparation for submission for publication).

Further studies are required to determine the exact mechanism of inhibition. Liu et al (2009) found that BMAA caused oxidative stress in foetal mouse cortical cell cultures by lowering of cystine uptake, a precursor for the intracellular antioxidant glutathione resulting in a decrease of that tripeptide in the cells, and led to accumulation of free radicals. However, this mechanism of induction of oxidative stress occurred at an exogenous BMAA concentration of 3 mM for 3 hours. In both studies, however, the cause of oxidative stress is indirect, either via inhibition of the antioxidant enzymes, as in our study or via inhibition of synthesis of an important antioxidant tripeptide, as evidenced by Liu et al. (2009).

4.5.3 Aquatic Macrophyte

Although Esterhuizen et al. (2011) noted no visible adverse effects in *C. demersum* plant sections after 24 hour exposure to BMAA, oxidative stress response enzymes were inhibited. Catalase and glutathione-*S*-transferase were significantly inhibited on exposure to 0.5 µg ℓ^{-1} BMAA while peroxidases, including glutatione peroxidase, superoxide dismutase and glutatione reductase were inhibited at exposure concentrations of 1 µg ℓ^{-1} and higher (Esterhuizen et al., 2011).

With the inhibition of the antioxidative stress enzymes on the addition of BMAA, an increase in ROS was observed with a 5 fold increase in ROS at 50 µg ℓ^{-1} BMAA. Esterhuizen et al., (2011) confirmed the inhibition of GR in vitro and showed that BMAA directly inhibits the enzyme.

Lobner et al. (2007) previously showed that one of the mechanisms by which BMAA is toxic is the production of ROS. The data represented here suggest that BMAA may in fact increase ROS simply by inhibiting the phase I and II oxidative stress enzymes. Additionally, GST, an enzyme required for detoxification by biotransformation via glutathione conjugation was also inhibited (Pflugmacher et al., 1998; Pflugmacher et al., 1999). Esterhuizen et al. (2011) therefore concluded that BMAA is not detoxified through phase II metabolism and that GST cannot be used as a biomarker for BMAA induced oxidative stress as GST activity was inhibited.

4.5.4 Terrestrial Macrophytes

The selected antioxidant defense enzymes CAT, GR, GPx, and SOD were assessed following BMAA exposure through the growth medium for seven days. BMAA was found to have an inhibitory effect on the activity of these enzymes for all the exposure concentrations, with exception for only one exposure concentration of 500 µg ℓ^{-1} for SOD. The enzyme activity for BMAA-treated plants was lower when compared to enzyme activity of control plants. There was dose-dependent decrease in enzyme activity (Figure 4.12).

There was a significant ($p = 0.0002$) effect of BMAA concentration on CAT activity for the

three concentrations tested. Post hoc comparisons using the Tukey HSD test indicated significant differences from controls (M = 84176.25, SD = 11749.54) in all treated groups: 100 µ µg ℓ^{-1} (M = 55836.31, SD = 11749.54), 300 µg ℓ^{-1} (M = 40731.39, SD = 15919.68) and 500 µg ℓ^{-1} (M = 35437.12, SD = 25867.91). There was no significant effect of any tested BMAA concentration on GR activity at the $p<0.05$ level for the three concentrations tested [$F(3, 26) = 2.38$, $p = 0.097$]. There was no significant effect of BMAA concentration on GPx activity at the $p<0.05$ level for the three concentrations tested [$F(3, 26) = 1.85$, $p = 0.162$].

There was a significant ($p = 0.0001$) effect of BMAA concentration on SOD activity. Post hoc comparisons using the Tukey HSD test indicated that the mean SOD activity for plants exposed to 100 µg ℓ^{-1} (M = 362.68, SD = 82.70) was not significantly different than the control plant SOD activity (M = 516.88, SD = 41.29). However, the activity in plants exposed to 300 µg ℓ^{-1} was significantly different to controls (M = 322.67, SD = 73.94)

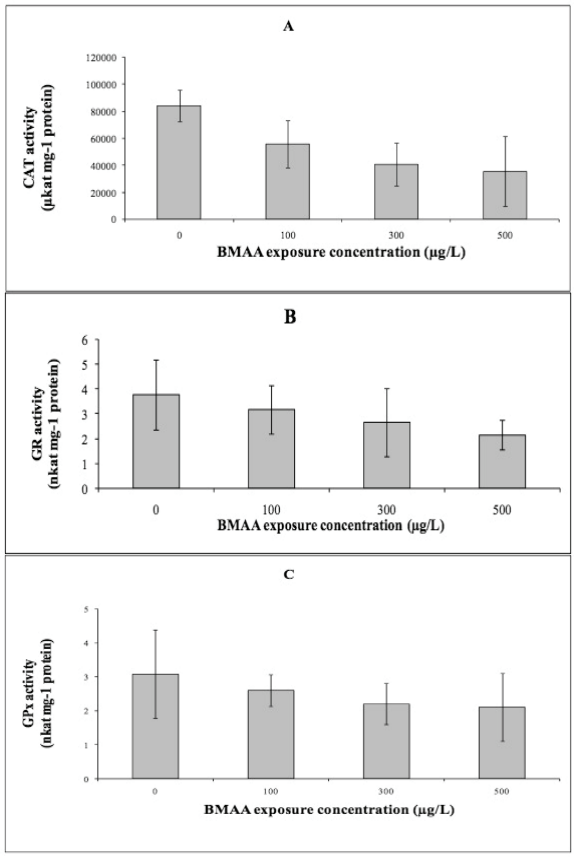

Figure 4.12. Activity of oxidative stress enzymes: (A) CAT [µkat mg^{-1} protein], (B) GR [nkat mg^{-1} protein], and (C) GPx [nkat mg^{-1} protein], extracted from *N. officinale*, after exposure to varying BMAA concentrations. Data are mean ± SD (n = 3). (Niyonzima, MSc dissertation, 2010. Article in preparation for submission for publication).

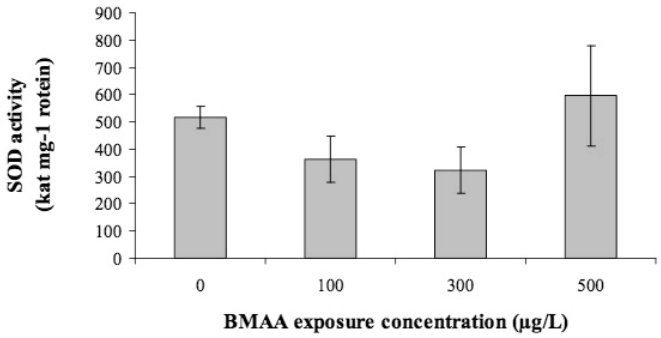

Figure 4.13. Activity of oxidative stress enzyme SOD [kat mg^{-1}protein], extracted from *N. officinale*, after exposure to varying BMAA concentrations. Data are mean ± SD (n = 4). (Niyonzima, MSc dissertation, 2010. Article in preparation for submission for publication).

4.6 Production of BMAA isotopes

Isotopically labeled BMAA was successfully synthesized using a proprietary method. The labeled product was verified using LC/MS/MS.

4.7 Metabolism of BMAA by model organisms

The re-distribution of the primary amino group and the methyl group were used to evaluate the modification or catabolism of BMAA by selected model organisms.

4.7.1 Cyanobacteria

Synechocystis PCC6803 was cultured in the presence of 100 µM L-BMAA-4,4,4-d_3,$^{15}N_2$. The redistribution of L-BMAA-4,4,4-d_3,$^{15}N_2$-originating stable isotopes to 13 amino acids (glu, gln, asp, asn, gly, trp, tyr, leu, ser, lys, orn and val) was monitored by LC/MS. Redistribution of stable isotopes 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.

As previously reported by Downing et al. (2012), extracellular BMAA is rapidly taken up by cyanobacteria. This was confirmed by the significant increase in free cellular labeled BMAA observed within the first hour of exposure. Intracellular BMAA levels continued to increase over the 48 hours exposure period with the rate of uptake decreasing after 24 hours. Three glutamate molecular isotopes, $^{1+}$glu, $^{2+}$glu and $^{3+}$glu, were detected and the change in the amounts of these glutamate isotopes over 48 hours exposure to BMAA-4,4,4-d_3,$^{15}N_2$ is depicted in Figure 4.14. These data show that free cellular glutamate, containing either 1,2 or 3 stable chemical isotopes, followed to same trend over the exposure period. This trend is marked by a gradual and significant decrease in $^{0+}$glu (147.13 $g.mol^{-1}$) within the first 24 hours, followed by a significant (p<0.05) increase in all detected glutamate molecular isotopes. When analyzing the redistribution of BMAA-4,4,4-d_3,$^{15}N_2$-originating stable isotopes throughout the free cellular amino acid pool, changes in the amounts of free cellular amino

acid isotopes can be misleading. This is due to the presence of naturally abundant stable chemical isotopes.

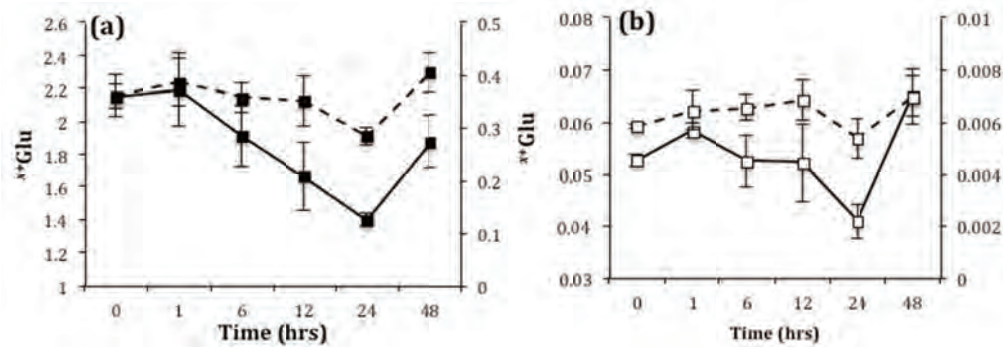

Figure 4.14. The change in free cellular glutamate over 48 hours exposure to BMAA-4,4,4-d_3,$^{15}N_2$, where (a) shows $^{0+}$glu (147.13 g.mol^{-1}) as a solid line and $^{1+}$glu (148.13 g.mol^{-1}) as broken line (secondary axis); and (b) shows $^{2+}$glu (148.13 g.mol^{-1}) as a solid line and $^{3+}$glu (148.13 g.mol^{-1}) as a broken line (secondary axis). Error bars denote standard deviation where n=3. (Article in preparation for submission for publication).

The natural abundance of nitrogen stable isotope is 0.368 percent, whereas ^{2}H does not occur naturally or in significant amounts (Rosman and Taylor, 1999). Due to the composition of cyanobacterial culture medium used, the natural abundance of these stable isotopes often differs from the aforementioned percentages and is routinely recorded at approximately 12 percent. All samples were normalized against the natural abundance of amino acid molecular isotopes determined based of amounts present at t0 (prior to the addition of BMAA-4,4,4-d_3,$^{15}N_2$). These data are presented in Figure 4.16 that shows the percentage change from t0 in glutamate stable isotopes relative to the entire free glutamate pool.

Although Figure 4.14 shows a decrease in the entire glutamate pool within 24 hours, 4.15(a) shows that the rate of decrease in $^{0+}$glu was greater than that of $^{1+}$glu, marked by the fact that the abundance (percentage) of $^{1+}$glu in the free glutamate pool increased significantly within the first 12 hours. This suggests that although the total amount of $^{1+}$glu decreased over the initial 24 hours exposure, continuing glu synthesis produced predominantly $^{1+}$glu resulting in a significant increase in the relative abundance of $^{1+}$glu within the cell. Figure 4.15(b) shows the proportional increase in $^{2+}$glu and $^{3+}$glu with a significant increase in cellular abundance following 12 hours of exposure, despite an overall decrease in amounts. The proportional increase in $^{2+}$glu and $^{3+}$glu was attributed to the increase in $^{1+}$glu by addition of a ^{15}N to pre-existing $^{1+}$glu and $^{2+}$glu at levels reflecting natural abundance. Thus an increase in $^{1+}$glu resulted in a proportional increase in $^{2+}$glu where the latter was observed at a level reflecting the natural abundance as observed in untreated samples.

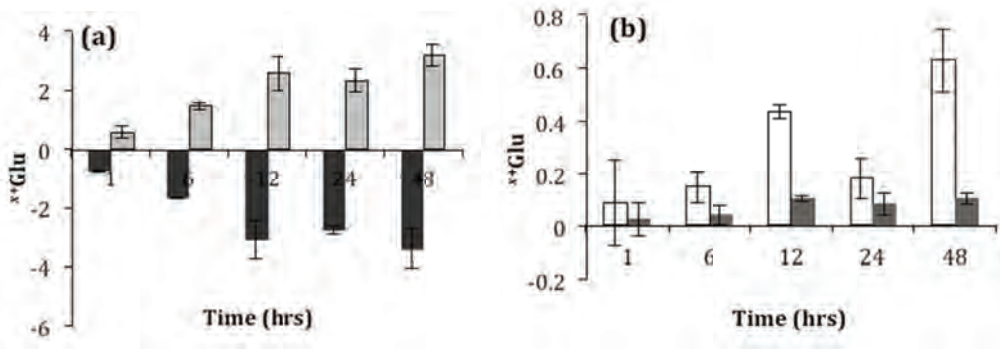

Figure 4.15. The change in free cellular glutamate isotopomers from their natural abundance at t0. (a) $^{0+}$glu (147.13 g.mol^{-1}) dark grey bars and $^{1+}$glu (148.13 g.mol^{-1}) light grey bars; and (b) $^{2+}$glu (148.13 g.mol^{-1}) open bars and $^{3+}$glu (148.13 g.mol^{-1}) light grey bars. Error bars denote standard deviation where n=3. (Article in preparation for submission for publication)

$^{2+}$glu and $^{3+}$glu, could either be due to the direct acquisition of BMAA-4,4,4-d_3,$^{15}N_2$-originating labels, or could simply reflect a proportional increase in the free cellular glutamate pool. For example; due to the presence of naturally abundant $^{1+}$glu already present in the cell prior to the addition of BMAA-4,4,4-d_3,$^{15}N_2$, an increase in $^{1+}$glu, due to the incorporation of BMAA-4,4,4-d_3,$^{15}N_2$-originating label, will automatically lead to an increase in $^{2+}$glu. The ratio of $^{2+}$glu: $^{1+}$glu would remain unchanged if the increase in $^{2+}$glu were solely due to an increase in $^{1+}$glu. An increase in $^{2+}$glu due to the incorporation of two BMAA-4,4,4-d_3,$^{15}N_2$-originating labels would result in an increase in the ratio, $^{2+}$glu: $^{1+}$glu. The same would hold true for any changes in $^{3+}$glu, $^{4+}$glu, etc.

Figure 4.16 shows the change in free cellular glutamate expressed as the percentage change in such ratios. These data show an increase in the ratio $^{1+}$glu:$^{0+}$glu (a) within the first hour of exposure to $^{5+}$BMAA and a further significant (p<0.05) increase over the following 12 hours. This data, together with that showed in Figure 4.16 suggests that $^{1+}$glu increased independently from $^{0+}$glu, meaning that one stable isotope label was redistributed to glutamate from $^{5+}$BMAA.

When considering the change in the abundance of $^{2+}$glu, data in Figure 4.16(b) show a significant (p<0.05) increase in $^{2+}$glu after 12 hours of exposure. However, when viewing these data in conjunction with data presented in Figure 4.16(b), which shows no significant change in the ratio of $^{2+}$glu: $^{1+}$glu in this time frame, it is evident that the initial increase in $^{2+}$glu is not due to the incorporation of two $^{5+}$BMAA-originating isotopes, but is simply due to an increase in $^{1+}$glu and the presence of naturally abundant $^{1+}$glu, as discussed above. The same holds true for $^{3+}$glu (data not shown). After 48 hours exposure however, there was a significant (p<0.05) increase in the abundance of $^{2+}$glu as well as a significant increase in the ratio of $^{2+}$glu: $^{1+}$glu. This suggests that after 48 hours in the presence of $^{5+}$BMAA, two

$^{5+}$BMAA-originating isotope labels were incorporated into free intracellular glutamate. No other significant increase in $^{x+}$glu (where x=3,4, or 5) were detected.

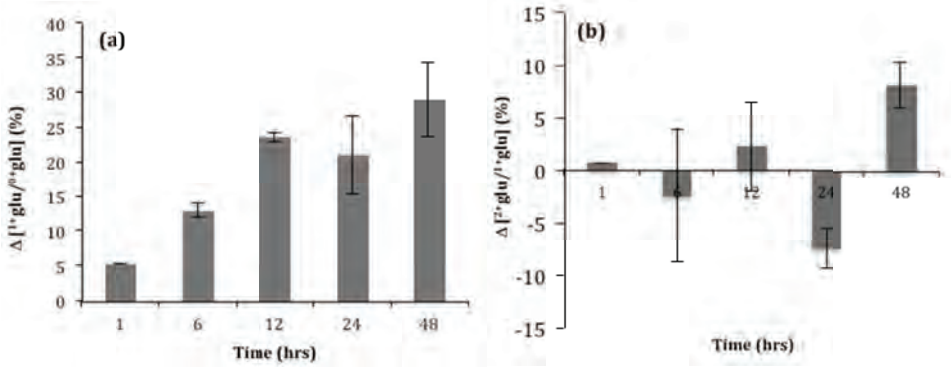

Figure 4.16. The change in free cellular $^{1+}$glu and $^{2+}$glu following exposure to $^{5+}$BMAA, expressed as a percentage change in the ratio of $^{1+}$glu: $^{0+}$glu (a) and $^{2+}$glu : $^{1+}$glu (b), respectively, with $^{x+}$glu at 0 hours taken as 100%. Error bars denote standard deviations where n=3. (Article in preparation for submission for publication)

In contrast to glutamate, free cellular glutamine increased over the 48 hours exposure period, shown in Figure 4.17(a), which indicates the absolute amounts of $^{0+}$gln and $^{1+}$gln. Only a single glutamine isotope, $^{1+}$gln, was detected with a natural abundance at t0 being below the limits of detection.

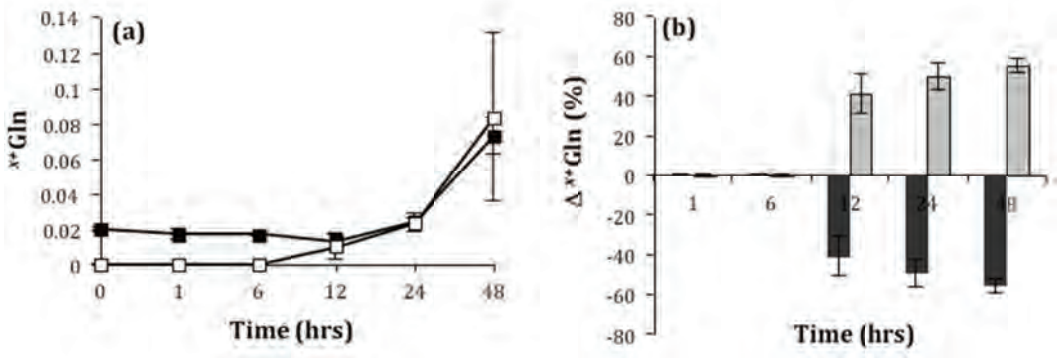

Figure 4.17. (a) The change in the absolute amounts of free cellular glutamine, where x=0, is 146.14 g mol^{-1} (solid squares) and x=1 is 147.14 g mol^{-1} (open squares) over 48 hours exposure to exogenous $^{5+}$BMAA. The graph depicts LC/MS peak areas normalized against internal standard, methionine-D3. (b) The change in free cellular glutamine molecular isotopes from their natural abundance at t0, $^{0+}$gln (146.14 g.mol^{-1}) dark grey bars and $^{1+}$glu (147.14 g mol^{-1}) light grey bars; and. Error bars depict standard deviation where n=3. (Article in preparation for submission for publication)

More remarkably, the abundance of $^{1+}$gln, relative to the entire free cellular glutamine pool, increased from 0 at 0-6 hours to approximately 56% 48 hr exposure, as seen in Figure 4.17(b). As discussed previously, in order to determine the nature of the change in the $^{1+}$gln

isotope one has to examine the change in the ratio of $^{1+}$gln: $^{0+}$gln, shown in Figure 4.18. The data presented in Figures 4.17 and 4.18 show that free cellular $^{1+}$gln increased significantly (p<0.05) and independently of an increase in $^{0+}$gln, following 12 hours of exposure to $^{5+}$BMAA, and continued to increase significantly (p<0.05) over the following 36 hours. This is important in determining the identity of the label that is responsible for the increases seen in $^{1+}$glu and $^{1+}$gln molecular isotopes.

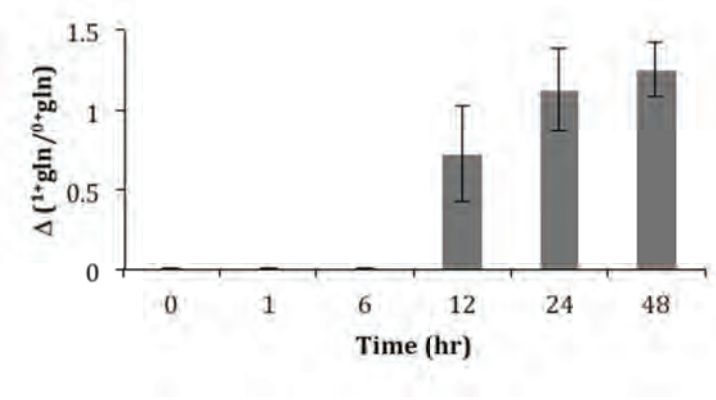

Figure 4.18. The change in free cellular $^{1+}$glu following exposure to $^{5+}$BMAA, expressed as the change in the ratio of $^{1+}$gln: $^{0+}$gln, normalized against the ratio at t0. Error bars denote standard deviations where n=3. (Article in preparation for submission for publication)

As the isotopically labeled BMAA contained 5 labels it is not possible to definitively identify the moiety that was redistributed to these two and other amino acids. The most likely isotope is the primary ^{15}N as transamination reactions involving primary amino groups are common and typically reversible. Unlike reactions involving secondary amino groups, primary amino transamination reactions form the basis of the majority of *de novo* amino acid synthesis reactions. Also possible but less likely is the redistribution of a single $^{1+}$proton within the cell. However, redistribution of individual ^{2}H would most likely be random and would not result in a clear and defined trend.

As discussed above data indicate that the two primary amino acids in *de novo* amino acid synthesis and nitrogen assimilation in cyanobacteria; glutamine and glutamate, both acquired at least one BMAA-4,4,4-d_3,$^{15}N_2$-originating isotope label within 12 hours of exposure to exogenous $^{5+}$BMAA. It is important to note that $^{1+}$glu increased within the first 1 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. Based on the correlation between data regarding the redistribution of the isotopes throughout *de novo* synthesized amino acids and the nature of nitrogen and/or amino acid metabolism in cyanobacteria, data suggest that it is the primary ^{15}N-amino group that may be responsible for the observed

increase in amino acid $^{1+}$isotopes.

Irrespective of the mode of BMAA biotransformation in cyanobacteria, the fact that cyanobacteria are biosynthesizers of BMAA must be kept in mind. The pathway of biotransformation undoubtedly offers insights into biosynthetic mechanisms but should not be considered a possible bioremediation option for BMAA. Rather, the rapid modification of BMAA supplied to cyanobacteria may equally well indicate the potential for rapid production of BMAA by a cyanobacterial bloom under certain environmental conditions.

4.7.2 Aquatic Macrophyte, *C. demersum*

Isotopically labeled BMAA (BMAA-4,4,4-d_3,$^{15}N_2$) was taken up from the culture medium by *C. demersum.* Figure 4.19 shows the change in the free intracellular BMAA pool over a 6-day period during which the plants were exposed to exogenous BMAA for the initial 24 hours and thereafter grew in BMAA-free media.

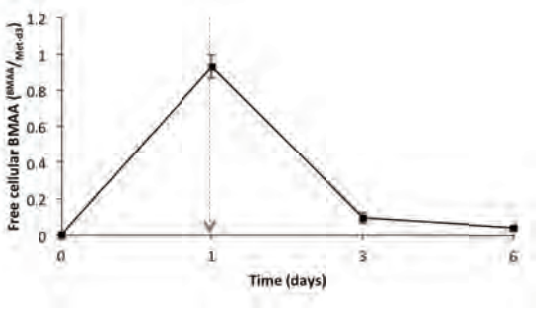

Figure 4.19. The change in the free cellular BMAA pool over a period of 6 days, initiated by the exposure of plants to exogenous BMAA-4,4,4-d_3,$^{15}N_2$ for 24 hours, followed by the transfer of plants into BMAA-free culture media (Arrow). Error bars depict standard deviations where n=3. (Article in preparation for submission for publication)

These data indicate the near complete removal of free cellular BMAA from the cell within 5 days. It is important to note that BMAA was not excreted or leaked back into the culture medium and that the decrease in free cellular BMAA is as a result of complete catabolism or modification of this amino acid. Only two molecular isotopes of BMAA were detected in samples; $^{5+}$BMAA and/or $^{4+}$BMAA.

Figure 4.20 shows the change in 5+ and 4+ BMAA isotopes expressed as a percentage of the total BMAA pool. These data show an increase (not significant) in $^{4+}$BMAA following exposure to exogenous labeled BMAA with a corresponding decrease in $^{5+}$BMAA, highlighted by a change in the ratio $^{4+}$BMAA: $^{5+}$BMAA shown in Figure 4.21. Although the amount of free $^{4+}$BMAA increased, evidenced by a significant (p<0.05) increase in the ratio $^{4+}$BMAA: $^{5+}$BMAA (Figure 4.21), it is important to note that the total increase $^{4+}$BMAA remained small at approximately 1%. This change, although notably low, could suggest an exchange of one

isotopically labeled atom for an unlabeled atom, such as exchange of the primary amino groups during transamination/amination reactions.

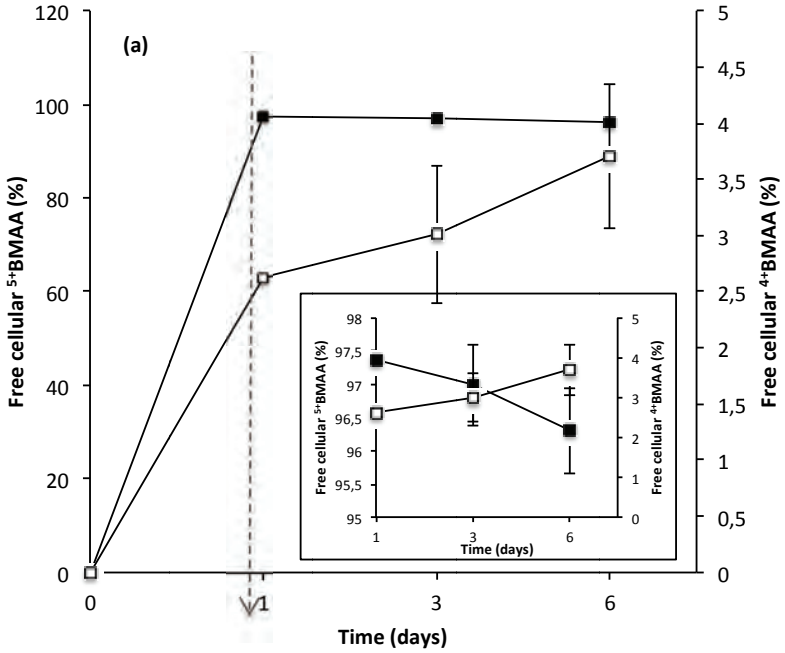

Figure 4.20. Intracellular BMAA molecular isotopes, expressed as a percentage of the total free intracellular BMAA pool. $^{5+}$BMAA (*m/z* = 338) solid squares, primary axis; $^{4+}$BMAA (*m/z* =337) open squares, secondary axis. Arrow indicates transfer into BMAA-free medium following 24 hours exposure to BMAA-4,4,4-d_3,$^{15}N_2$. (Insert) Highlights the change in BMAA molecular isotopes following transfer into BMAA-free media. Error bars denote standard deviations where n=3. (Article in preparation for submission for publication)

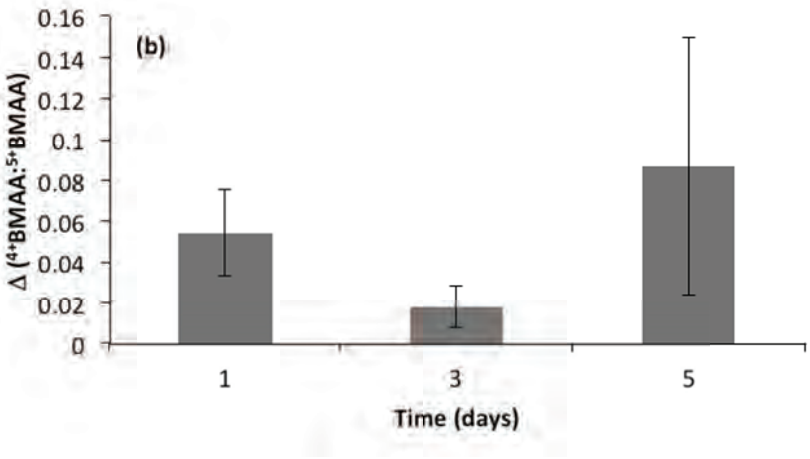

Figure 4.21. The change in the ratio $^{4+}$BMAA:$^{5+}$BMAA, expressed as the difference between this ratio in plants not exposed to $^{5+}$BMAA and plants exposed to $^{5+}$BMAA over 5 days. Error bars denote standard deviations where n=3. (Article in preparation for submission for publication)

These data suggest that *Ceratophyllum* metabolizes BMAA. In order to establish possible pathways of BMAA metabolism, redistribution of stable isotopes to other amino acids was investigated through analyzing the changes in molecular isotopes of glutamate and glutamine, two central molecules in amino acid metabolism.

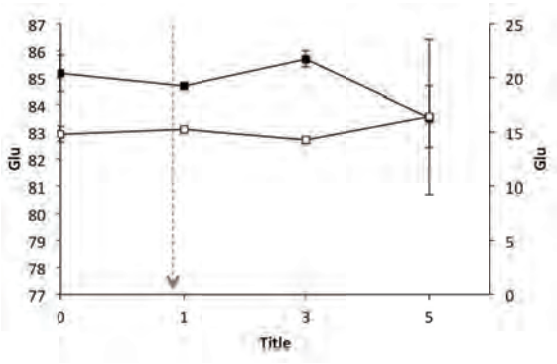

Figure 4.22. The change in free cellular glutamate molecular isotopes over 6 days, expressed as a percentage of the total free cellular glutamate pool. Arrow indicates placement of plants into BMAA free culture media following 24 hours exposure to $^{5+}$BMAA. $^{0+}$Glu, solid squares, primary axis and $^{1+}$Glu open squares, secondary axis. Error bars depict standard deviations where n=3. (Article in preparation for submission for publication)

Two glutamate molecular isotopes were detected; +0 and +1, however, no significant changes in these glutamate isotopes were recorded over the 6 day period (Figure 4.22). Three glutamine molecular isotopes were detected; +0, +1, and +2, with a significant (p<0.05) change seen only in the +2 isotope, which increased significantly within the initial 24 hour exposure to $^{5+}$BMAA (Figure 4.23).

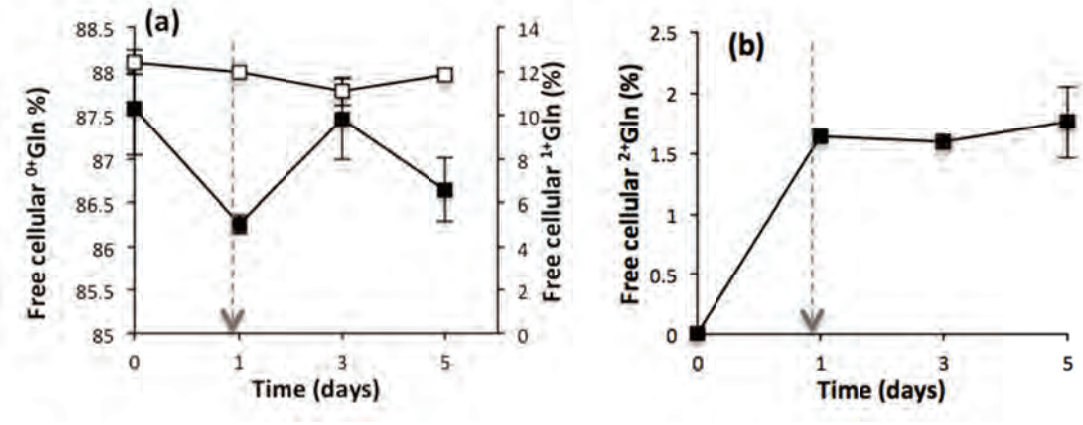

Figure 4.23. The change in free cellular glutamine molecular isotopes over 6 days, expressed as a percentage of the total free cellular glutamine pool. Arrow indicates placement of plants into BMAA free culture media following 24 hours exposure to $^{5+}$BMAA. (a) $^{0+}$Gln, solid squares, primary axis and $^{1+}$Gln open squares, secondary axis; (b) $^{2+}$Gln. Error bars depict standard deviations where n=3. (Article in preparation)

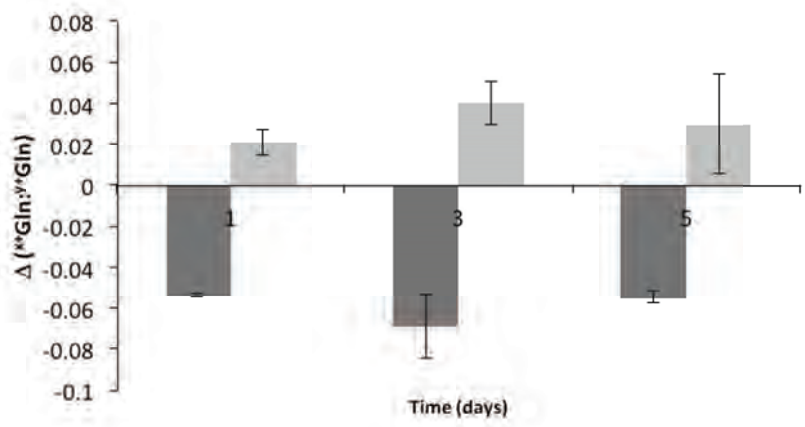

Figure 4.24. The change in the ratio $^{1+}$Gln:$^{0+}$Gln (dark grey barks) and $^{2+}$Gln:$^{1+}$Gln (light grey bars) relative to the natural abundance present in *Ceratophyllum* prior to exposure to $^{5+}$BMAA. Error bars depict standard deviations where n=3. (Article in preparation for submission for publication).

The data presented in Figure 4.23 and Figure 4.24 show that the $^{2+}$glutamine isotope increased following 48 hours (24 hours in the presence of BMAA and 24 hours in BMAA-free media) exposure to $^{5+}$BMAA. The increase in the ratio $^{2+}$Gln:$^{1+}$Gln at 1 and 3 days following 24 hours exposure to $^{5+}$BMAA suggests that this increase in 2+gln was independent from any changes in the abundance of the $^{1+}$gln isotope. No significant changes in any glutamate isotopes were detected. Other amino acids in which an increase in $^{1+}$isotopes were observed include arg, ser, and lys and where an increase in $^{2+}$isotopes were recorded in ser, asn, lys, and asp, with aspartate also showing a significant (p<0.05) increase in its $^{3+}$isotope. However the maximum increase in all of these changes in amino acid isotopes was less than 3% and occurred only after a minimum of 48 hours following exposure to $^{5+}$BMAA, with some changes only occurring after 5 days. This suggests that *Ceratophyllum* does not metabolise BMAA via primary amino acid metabolic pathways. Redistribution of $^{5+}$BMAA-originating isotopes to free cellular amino acids most likely occurred after catabolism of BMAA or at least the removal of the primary amine, which resulted in the dissemination of this chemical group, that was then free to be used in other anabolic processes such as amino acids biosynthesis.

4.7.3 Mussels

In order to determine whether or not BMAA is metabolized by these mussel species, the redistribution of stable isotopes throughout proteinogenic amino acids following exposure to L-BMAA-4,4,4-d_3,$^{15}N_2$ was investigated. Data showed no significant increase in glutamate or glutamine molecular isotopes and where increases were recorded such increases were minor and could not explain the large discrepancy between BMAA present in mussel tissue and BMAA missing from the culture medium. Catabolism of such quantities of BMAA would have resulted in significant increases in isotopes of several amino acids as is observed in cyanobacteria and *Ceratophylum*. Therefore, data from these studies suggest that the

neurotoxin is taken up by selected freshwater mussels but is not metabolized through regular amino acid metabolic pathways. Instead, BMAA is accumulated from the environment and appears to be reversibly covalently modified. This information impacts significantly on the assessment of the risk of human exposure to this neurotoxin and provides important insights for the formulation of human risk exposure guidelines and the control and prevention of human exposure to this toxin.

5 CONCLUSIONS

Cyanobacteria were shown to produce BMAA from raw materials confirming that cyanobacteria are the source of environmental BMAA and putting an end to the lengthy controversy regarding the origin of environmental BMAA. This highlights the potential risk of exposure to the toxin during mass proliferation of cyanobacteria in fresh waters. Typical water treatments were, however, shown to be effective at laboratory scale in removing BMAA, demonstrating that treated water poses little risk for BMAA exposure. However, the potential for exposure via recreational use of surface waters with significant cyanobacterial biomass, or via consumption of raw water containing cyanobacteria remain a concern. This report also confirms the possibility of bioaccumulation of the toxin in freshwater ecosystem components that may in turn be ingested by humans. The use of a range of model organisms and one natural system to show the bioaccumulation and transfer through trophic levels suggests that BMAA uptake and transfer through trophic levels may be common in freshwater systems, and human exposure therefore equally common. Given the frequency of recreational use of freshwater impoundments, the propensity to site residential areas near such impoundments where possible, and the sport and commercial use of fish resources, the bioaccumulation and possible biomagnification of BMAA indicate a real and present risk of human exposure to BMAA. In order to quantify this risk, a site-specific long-term study aimed specifically at evaluating human exposure risk at that site is indicated.

The uptake of BMAA by many organisms demonstrated here also offers the potential for bioremediation of BMAA contaminated waters. Where BMAA can be accumulated in non-food plants, this offers the potential for BMAA removal by large-scale use of such plants. Furthermore, where an organism has the potential for biotransformation, such an organism may remain in the ecosystem without posing a threat via biomagnification of accumulated BMAA. Given the general toxicity observed in all model organisms, the potential for ecological impact upon lysis of a large quantity of cyanobacterial biomass should also be considered, particularly where bioremediation is relied upon to detoxify or trap BMAA. The data presented here form the basis for evaluation of native species for the implementation of a so-called green liver system specifically designed to treat toxic water contaminants based on the use of tolerant indigenous organisms capable of biotransforming the toxin or accumulating it to high levels and having a non-food and non-agricultural application for the biomass thus produced. It is highly recommended that appropriate indigenous aquatic macrophytes be screened for biotransformation potential and those that show appropriate metabolic activities, used in mass culture to evaluate their potential for BMAA removal.

6 RECOMMENDATIONS

Given the potential for human exposure demonstrated here, it is recommended that the actual human exposure be evaluated at selected sites and that specific ecosystems and specific impoundments are studied with respect to the biotransformation and/or bioaccumulation of BMAA. The evaluation should include a long-term study of cyanobacterial biomass in an impoundment and BMAA levels in this biomass and that of zooplankton, green algae, macrophytes, invertebrates and vertebrates, so as to establish the time frame of food web distribution.

The finding that many organisms take BMAA up and that some are able to catabolize the toxin forms the basis for the evaluation of endemic plant species for either “floating island” or “green liver” in situ remediation systems. It is therefore recommended that suitable endemic aquatic macrophytes be evaluated for their ability to catabolize BMAA, and that the potential for use of these endemic plants in a “green liver” or “floating island” be established.

No specific steps to reduce possible exposure risk are, as yet, indicated. The practice of posting warning notices in the event of cyanobacterial blooms should continue in the absence of knowledge on aerosol exposure and actual risk of ingestion of fish and/or other aquatic organisms. These risks should be evaluated in a study of selected impoundments prone to cyanobacterial blooms, wherein aerosol exposure by nearby residents, recreational users and any commercial activity be evaluated. Linked to this, an epidemiological study should be initiated where a food item shown to accumulate BMAA and sourced from that impoundment, is usually available, and the transfer of BMAA top consumers can be evaluated.

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