BIOACCUMULATION AND ECOTOXICOLOGY OF
β-METHYLAMINO-L-ALANINE (BMAA) IN
MODEL CROP PLANTS

by

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DECLARATION

I, Francois Niyongabo Niyonzima, s207049125, hereby declare that the dissertation for the degree Magister Scientiae is my own work and that it has not previously been submitted for assessment or completion of any postgraduate qualification to another University or for another qualification.

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Cyanobacteria are known to produce a variety of toxic compounds. \( \beta \)-\( N \)-methylamino-L-alanine (BMAA) is one of the neurotoxins produced by most cyanobacteria. BMAA has been implicated in amyotrophic lateral sclerosis / Parkinsonism dementia complex (ALS / PDC) and was suggested to contribute to this pathology after biomagnification and slow release of BMAA from a protein associated form. The uptake and accumulation of BMAA by the aquatic macrophyte *Ceratophyllum demersum* has recently been shown, but the consumption of aquatic macrophytes by humans is not typical. The uptake by, and accumulation in, crop plants (*Nasturtium officinale* and *Daucus carota*) was therefore investigated so as to establish the existence of any risk to humans from the consumption of plants irrigated with water from dams with high cyanobacterial biomass and therefore high BMAA levels. After the exposure to the BMAA through the growth medium, BMAA had no effect on growth and development of *N. officinale* and *D. carota*. The uptake and bioaccumulation of BMAA was observed in *N. officinale* and *D. carota*, and was found to be concentration-dependent. Both free and bound cellular BMAA was detected following BMAA exposure through the growth medium. The photosynthetic apparatus of *N. officinale* was not significantly damaged. The uptake and accumulation of BMAA in edible terrestrial plants may constitute another route of human exposure to BMAA; it may now be prudent to avoid spray irrigation of edible plants with waters from dams with high cyanobacterial biomass and therefore high BMAA levels. After uptake by plants, the cyanotoxins may induce oxidative stress. A recent study showed that BMAA has a significant inhibitory effect on the oxidative stress enzymes in *C. demersum*. Therefore, the toxicological effects on selected plants were investigated by a range of biochemical enzyme assays in order to establish the plant stress response to exogenous BMAA. The inhibition of antioxidant enzymes upon exposure of *N. officinale* to BMAA through the growth medium was observed. The inhibition of antioxidant defence enzymes by BMAA correlated with the BMAA bioaccumulation in *N. officinale*. Further investigations are needed to analyze the uptake, accumulation, and ecotoxicology of BMAA in other crop plants, and to examine the fate of BMAA in these plants particularly its distribution and metabolism.
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LIST OF ABBREVIATIONS

AD: Alzheimer’s disease
ALS/PDC: amyotrophic lateral sclerosis / Parkinsonism dementia complex
amu: atomic mass unit
APx: ascorbate peroxidase
AQC: 6-aminoquinolyl-N-hydrosuccinimidyl carbamate
AtGLR: Arabidopsis glutamate receptor-like
BMAA: β-N-methylamino-L-alanine
BSA: Bovine serum albumin
CAT: catalase
DNA: deoxyribonucleic acid
DTE: dithioerythritol
DW: dry weight
EAA: excitatory amino acid (EAA)
EDTA: ethylenediaminetetraacetic acid
Fig.: figure
Fm: maximum fluorescence intensity
Fo: basal fluorescence intensity
Fv / Fm: maximal photochemical efficiency
GPx: glutathione peroxidase
GR: glutathione reductase
GSH: reduced glutathione
GSSG: glutathione disulfide (oxidized glutathione)
GST: glutathione S-transferase
HARG: homoarginine
HPHE: homophenylalanine
kat: katal
L: liter
LPO: lipid peroxidation
LC-MS: liquid chromatographic mass spectrometry
M: molar
MC: microcystin
MC-LR: microcystins-LR
MDHA: monodehydroascorbate
Met: methionine
mg: milligram
mGluR5: metabotropic glutamate receptor 5
min: minute
mM: millimolar
NADP+: oxidised nicotinamide adenine dinucleotide phosphate
NADPH: reduced nicotinamide adenine dinucleotide phosphate
nkat: nano katal
POD: peroxidases
PSII: photosystem II
RNA: ribonucleic acid
ROS: reactive oxygen species
Rt: retention time
Sec: second
SOD: superoxide dismutase
STD: standard
SD: standard deviation
Temp: temperature
Trp: Tryptophan
U: units
WST: water-soluble tetrazolium salt
ε: molar extinction coefficient
1 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Cyanobacteria are photosynthetic prokaryotes that may produce a variety of toxic compounds including neurotoxins, hepatotoxins, cytotoxins, and lipopolysaccharide endotoxins. These can pose a serious threat to human and animal health (Carmichael, 1997). β-N-methylamino-l-alanine (BMAA) is one of the neurotoxins produced by most cyanobacteria from marine, freshwater, brackish aquatic environments, and terrestrial environments (Cox et al., 2005; Banack et al., 2007; Esterhuizen and Downing, 2008). BMAA can therefore, like cyanobacteria, have a global distribution, and the occurrence of BMMA may be a widespread phenomenon (Jonasson et al., 2008; Metacalf et al., 2008). BMAA has been implicated in amyotrophic lateral sclerosis / Parkinsonism dementia complex (ALS / PDC) and was suggested to cause or contribute to this pathology after biomagnification and slow release of BMAA from a protein associated form (Murch et al., 2004).

Hazard characterization and risk assessment of cyanobacterial toxins in relation to human health requires the identification of exposure routes. One of these routes is the consumption of organisms that have accumulated BMAA (Murch et al., 2004). Another route may be the consumption of foods previously exposed to cyanotoxins. For instance, crop plants can be associated with cyanobacterial cells or toxins after spray irrigation with water which had experienced a cyanobacterial bloom. For example, microcystins were retained by lettuce plants (Lactuca sativa) after cultivation under spray irrigation with water containing Microcystis aeruginosa (Codd et al., 1999). The exposure of plants to microcystins through the growth medium also has an adverse effect on development (Abe et al., 1996; McElhiney et al., 2001; Gehringer et al., 2003; Pflugmacher et al., 2006). Therefore, the accumulation of the cyanotoxins in edible plant tissues may provide a route for human intoxication as the cyanotoxins can be carried through the food chain (McElhiney et al., 2001).
Recent studies by Metcalf et al. (2008) showed that BMAA is present in cyanobacterial blooms and mats and may therefore also be released into freshwater lakes and brackish water bodies used by humans. In these biomass samples, BMAA was found to co-occur with other cyanobacterial toxins including microcystins, nodularin, anatoxin-a, and saxitoxins. The uptake and accumulation of BMAA by the aquatic macrophyte *Ceratophyllum demersum* has recently been shown by Esterhuizen et al. (2009, personal communication), but the consumption of aquatic macrophytes by humans is not typical. It is therefore necessary to determine the uptake by, and accumulation in, terrestrial plants so as to establish the existence of any risk to humans from the consumption of plants irrigated with water from dams with high cyanobacterial biomass and therefore high BMAA levels. If BMAA can bioaccumulate in terrestrial plants, the effects on these plants can also be evaluated.

Terrestrial plants can come into contact with cyanotoxins via irrigation (McElhiney et al., 2001). After uptake by plants, the cyanotoxins may induce oxidative stress via the enhanced production of reactive oxygen species (ROS) (Pflugmacher, 2004; Pflugmacher et al., 2006). If the initiated oxidative stress is not stopped, extensive cellular damage such as DNA damage, protein inhibition or lipid peroxidation may occur (Pflugmacher, 2004). A recent study by Esterhuizen et al. (personal communication) showed that BMAA has a significant inhibitory effect on the oxidative stress enzymes in *C. demersum*. Therefore, the toxicological effects on selected plants can be evaluated by a range of biochemical enzyme assays in order to establish the plant stress response to exogenous BMAA.

### 1.2 Cyanobacterial toxins

The production of cyanotoxins is a well-known feature of cyanobacteria, and in many cases has been connected with lethal health effects (Carmichael, 1997). Many of the bloom-forming cyanobacteria are known to produce several different toxins including neurotoxins, hepatotoxins, cytotoxins, lipopolysaccharide endotoxins (irritant toxins), and dermatotoxins (Carmichael, 1997).
Cyanotoxins can be grouped based on the physiological systems, organs, tissues, or cells that are primarily affected. They include:

Cytotoxins: These include cylindrospermopsin, a guanidine alkaloid capable of inhibiting protein synthesis, causing widespread necrotic injury in mammals, chromosome loss, and DNA strand breakage (Humpage et al., 2000).

Hepatotoxins: These include cyclic heptapeptide microcystins, and the cyclic pentapeptide nodularins. These peptides inhibit protein phosphatases, trigger changes in membrane integrity and are tumour promoters. In addition, nodularin is also carcinogenic (Carmichael, 1997; Falconer, 1998; Codd, 2000). Microcystins are produced by *Microcystis*, *Anabaena*, *Planktothrix*, *Hapalosiphon* and *Nostoc*, and are potent inhibitors of plant and animal phosphatases 1 and 2A, which play a pivotal role in cellular processes like cell growth (Codd, 2000). The accumulation of chlorophyll, a key pigment involved in plant photosynthesis, is also inhibited by microcystins (McElhiney et al., 2001).

Irritants and gastrointestinal toxins: These include aphysatoxin, debromoaphysiatoxin, and lyngbyatoxin, which are produced by marine cyanobacteria, cause skin irritation, and are tumour promoters; and lipopolysaccharide endotoxins, which may trigger inflammatory and gastrointestinal incidents (Falconer, 1998; Codd, 2000).

Neurotoxins: These include alkaloids anataxin-a, and homoanatoxin-a, which are postsynaptic, cholinergic neuromuscular blocking agents; anatoxin-a (S), a guanidine methyl phosphate ester which inhibits acetylcholinesterase; and saxitoxins, carbamate alkaloids that block sodium channels (Carmichael, 1997; Falconer, 1998; Codd, 2000).

Certain species of cyanobacteria such as *Anabaena*, *Aphanizomenon*, *Oscillatoria*, *Lyngbya*, and *Cylindrospermopsis*, are known to produce various neurotoxins including anatoxin-a, homoanatoxin-a, anatoxin-a (S), and saxitoxins (Codd, 2000), whereas the neurotoxin BMAA was found to be produced by most cyanobacteria (Cox et al., 2005).
1.3 BMAA

BMAA (Fig. 1.1) is a neurotoxic non-proteinogenic amino acid produced by the majority of cyanobacteria (Cox et al., 2005). It has a mass of 118.13 amu. It is not lipophilic but water soluble (Jonasson et al., 2008). It is a neutral amino acid at physiological pH values (Nunn and O’Brien, 1989).

![Structure of BMAA](image)

**Figure 1.1**: Structure of BMAA (synonyms found in literature: 2-amino-3-methylamino-propanoic acid; L-α-amino-β-methylaminopropionic acid) (Jonasson et al., 2008).

BMAA was discovered in 1967 by Vega and Bell in the seeds of the cycad *Cycas micronesica* that lives in symbiotic association with cyanobacterium *Nostoc* (Jonasson et al., 2008). It can occur both in free and protein-bound forms (Murch et al., 2004), and was thought to be synthesized from α-acetamidoacrylic acid (Vega and Bell, 1967). It has a relatively high chemical stability, because unchanged BMAA has been detected in tissues of flying foxes and human brain (Banack and Cox, 2003; Banack et al., 2006; Jonasson et al., 2008).

Brenner et al. (2003) proposed a two-step pathway for the biosynthesis of BMAA in cycads, based on analysis of expressed sequence tags, obtained from RNAs of young *Cycas rumphii* leaves. In this pathway, BMAA synthesis starts with the transfer of NH$_3$ to β-substituted alanine to form a metabolic intermediate. A cysteine synthase-like enzyme catalyzes this reaction. The second step involves the transfer of a methyl group from S-adenosylmethionine (Ad-S-CH$_3$) to the new amine group of the intermediate compound, and is catalyzed by a methyltransferase (Fig. 1.2).
BMAA requires derivatisation in order to be detected with either fluorescence or by UV-VIS. Due to its stability and rapid reaction rate, 6-aminoquinolyl-N-hydrosuccinimidyl carbamate (AQC) was found to be a good derivatizing agent. The derivatizing agent reacts with primary and secondary amines leading to the formation of fluorescent derivates (Murch et al., 2004; Cox et al., 2005; Banack at al., 2006). It is difficult to obtain accurate results by using fluorescence detection after derivatisation because of contaminants, including amino acids present in the environmental samples or other cyanobacterial extracts (Cox et al., 2005); therefore, all data from analytical methods must be supported with additional data from a complimentary detection technique, for example liquid chromatographic mass spectrometry (LC-MS) (Murch et al., 2004; Cox et al., 2005; Banack et al., 2007; Esterhuizen and Downing, 2008).

The ecological function of BMAA in cyanobacteria and in the cycads remains to be elucidated (Jonasson et al., 2008) however, Cox et al. (2003) suggest that BMAA in cycads may function as a chemical deterrent to herbivory because the high concentration of BMAA found in the developing reproductive organs of C. micronesica, specifically in the immature male sporangia (1,564 µg/g) and in the outer integument layer of the seed sarcotesta. In addition, since each molecule of BMAA has two nitrogen atoms (Fig. 1.1) suggesting BMAA seems to play a minor role as a mechanism of nitrogen transfer within the coralloid root / cyanobacterial symbiosis (Cox et al., 2003). BMAA might serve as nitrogen source since amino acids are used by the plants as nitrogen source (Syennerstam et al., 2007).
1.4 BMAA biomagnification

BMAA can be associated with or incorporated into proteins (Murch et al., 2004), as are many other non-protein amino acids (Rosenthal, 1977; Neilan et al., 1999). BMAA has been artificially incorporated into synthetic peptides (Seebach et al., 1994) and it can be released from a protein associated or protein bound form by acid hydrolysis (Murch et al., 2004). The ratio between the protein bound and free BMAA is between 60:1 and 120:1 (Ince and Codd, 2005). The bound form of BMAA may function as an endogenous neurotoxic reservoir that can slowly release free BMAA directly into the brain tissues through protein metabolism (Fig. 1.3), thereby causing neurological damage over years or even decades (Murch et al., 2004; Banack et al., 2006). This increases the potential health risk even for organisms exposed to low doses of BMAA, and would explain its non-acute mode of toxic action (Murch et al., 2004; Ince and Codd, 2005).

Figure 1.3: The endogenous neurotoxic reservoir (Murch et al., 2004).

In the Guamanian ecosystem, BMAA is produced by cyanobacteria of the genus Nostoc, (which are root symbionts of cycads), and is then biomagnified as it travels through the food chain from cyanobacterial symbionts in cycad plants, to the flying foxes (Pteropus mariannus, Pteropodidae) that forage on the cycad seeds (Fig. 1.4), and then to the people who eat the bats (Cox et al., 2003).
Figure 1.4: Cycas micronesica Hill. (a) Habitat in South Guam as a 4-m-tall unbranched tree. (b) Positively geotropic coralloid roots with tips cut to show zone of cyanobacterial invasion. (c) Cross section of coralloid root showing green ring of cyanobacterial growth. (d) Pteropus mariannus feeding on fleshy sarcotesta of seed. (e) Cyanobacteria of the genus Nostoc cultured from the coralloid roots (Cox et al., 2003).

Free-living cyanobacteria (Nostoc) produce 0.3 µg.g⁻¹ BMAA (Cox et al., 2003), but BMAA occurs at 37 µg.g⁻¹ in the coralloid roots of cycad trees, and at 3,556 µg.g⁻¹ in the flying foxes that forage on the sarcotesta of Cycas micronesica seeds (Banack and Cox, 2003; Cox et al., 2003). The concentrations of BMAA in the flying foxes were derived from 50-year-old museum samples (Banack and Cox, 2003), providing an indication of the stability of the BMAA-protein complex (Murch et al., 2004). Thus, the biomagnification of BMAA through the Guam ecosystem fits a classical triangle of increasing concentrations of the toxic compounds up the food chain (Fig. 1.5) (Cox et al., 2003; Jonasson et al., 2008).
Figure 1.5: Biomagnification of cyanobacterial BMAA in Guam. The widths of the arrows are proportional to the concentration of free BMAA delivered to the next higher trophic level (Cox et al., 2003).

No correlation was noticed between the free or bound BMAA concentrations within or between taxonomic groups or geographic location (Cox et al., 2005; Esterhuizen and Downing, 2008).

1.5 BMAA neurotoxicity

BMAA has been found to be involved in the development of neurological diseases, such as ALS/PDC and Alzheimer’s disease (AD) (Murch et al., 2004). BMAA was suggested as a possible causative agent of the deadly neurodegenerative disease, ALS/PDC, common among the Chamorro people of Guam, because:

Firstly, BMAA was shown to be magnified as it travels through the food chain from cyanobacterial symbionts on cycad plants, to the flying foxes, and to people who eat the bats (Cox et al., 2003).
Secondly, studies undertaken by Monson et al. (2003) have shown a sudden decline in ALS/PDC occurrence on Guam with a decline in the consumption of flying foxes.

Thirdly, BMAA can be associated with or incorporated into proteins (Murch et al., 2004), as do many other non-protein amino acids. This association may lead to further increases in human BMAA levels and provide a mechanism for slow release, which accounts for the delayed onset of ALS/PDC following the time of probable BMAA consumption (Ince and Codd, 2005).

Fourthly, BMAA has been found in the cyanobacteria present throughout the world (Cox et al., 2005; Esterhuizen and Downing, 2008), suggesting that BMAA may be of concern not only for the Chamorro people, but also for the entire population of the planet. Murch et al. (2004) reported that BMAA is not only found in the brain samples of ALS/PDC patients from the Guam, but also in Alzheimer’s disease patients from Canada. Therefore, since the cycads are not part of the Canadians flora, the cyanobacteria might be the ultimate source of the BMAA in the Canadian Alzheimer’s patients (Cox et al., 2003).

Although BMAA is the most likely candidate for ALS/PDC in Guam, other neurotoxic cycad molecules such as cycasin (methylazoxymethanol β-D-glucoside), sterol-D-glucoside or other unknown compounds, might be similarly biomagnified and therefore should also be considered as possible environmental neurotoxins related to ALS/PDC (Khabazian et al., 2002; Cox and Banack, 2006). For instance, cycasin extracted from Chamorro cycad flours was approximately 10 times higher than that of BMAA, and the largest concentrations of cycasin were found in samples from villages with a high reported prevalence of ALS/PDC (Kisby et al., 1992). In addition, increased concentrations of BMAA were also found from the fieldwork on the island of Guam (Banack et al., 2006). Furthermore, studies in mice have indicated that administration of BMAA does not induce neuronal death in vivo (Perry et al., 1989; Cruz-Aguado et al., 2006). Montine et al. (2005) also found no evidence of BMAA in brains of patients diagnosed with ALS/PDC or AD.
BMAA has been found to cause selective death of motor neurons in vitro at concentrations as low as 30 µM, by acting to kainate receptors. An increase in intracellular calcium concentration and ROS production was also observed. This suggested a largely oxidative mechanism of injury (Rao et al., 2006). Lobner et al. (2007) also reported that BMAA can cause selective death of motor neurons in vitro at concentrations as low as 10 µM. Induction of oxidative stress and changed protein function via the incorporation of BMAA into proteins have thus been suggested as mechanisms for BMAA toxicity (Murch et al., 2004; Lobner, et al., 2007).

1.6 BMAA ecotoxicology

Terrestrial plants are capable of taking up cyanotoxins in sufficient amounts to induce morphological changes (McElhiney et al., 2001; Pflugmacher et al., 2006) and also physiological changes (Gehringer et al., 2003) in plants. After uptake, cyanotoxins may affect the early state of development such as seed germination, primary root length, or photosynthesis (Abe et al., 1996; McElhiney et al., 2001; Pflugmacher et al., 2006). BMAA was found to inhibit both root growth and shoot growth during early seedling development of Arabidopsis, in a glutamate-reversible manner (Brenner et al, 2000). Studies undertaken by Gehringer et al. (2003) showed an important decrease of leaf length in Lepidium sativum after exposure to 1 µg/L or 10 µg/L MC-LR; simultaneously glutathione S-transferase activity was also significantly raised from days four and five.

1.6.1 Chlorophyll fluorescence

Leaves contain chlorophyll molecules capable of absorbing light energy that can have one of three fates: it can be used to drive photosynthesis (photochemistry), excess energy can be dissipated as heat, or it can be re-emitted as radiation (chlorophyll fluorescence). These three processes occur in competition so that any increase in the efficiency of one will result in a decrease in the yield of the other two. The information about changes in
the efficiency of photochemistry and heat dissipation can therefore be obtained by measuring the yield of chlorophyll fluorescence (Maxwell and Johnson, 2000).

The first important realization of the relationship between primary reactions of photosynthesis and chlorophyll \(a\) fluorescence came from Kaustky and Hirsh (1931). They reported that upon transferring photosynthetic material from the dark into the light, the chlorophyll \(a\) fluorescence emission is not constant, but exhibits a fast increase to a maximum over a time period of around 1 second, followed by a decline to reach a steady level. They postulated that the rising phase of this transient reflected the primary reactions of photosynthesis, whereas the declining phase correlated with an increase in the \(\text{CO}_2\) assimilation.

Chlorophyll fluorescence was found to be a valuable non-invasive, highly sensitive, fast and easy technique to study the ability of a plant to tolerate environmental stresses and to what extent to which those stresses have damaged the photosynthetic apparatus (Strasser et al., 1995). For example, when used as a simple detector of biotic stress induced by microcystins in aquatic plants \(\text{Lemna gibba}\), the maximal photochemical efficiency (Fv/Fm) was time and microcystin concentration dependent (Saqrane et al., 2009). Järvenpää et al. (2007) applied chlorophyll fluorescence to evaluate the physiological state of the photosynthetic apparatus in the terrestrial plants, \(\text{Sinapis alba}\) and \(\text{Brassica oleracea}\) var. \(\text{italica}\) exposed to various concentrations of microcystins (MC-LR).

Dark-adapted values of Fv/Fm ratios, obtained by the measured basal fluorescence (Fo) and maximum fluorescence (Fm) emission \([\text{Fv/Fm} = (\text{Fm}-\text{Fo})/\text{Fm}]\), reflected the potential quantum efficiency of photosystem II (PSII), and are thus used as sensitive indicator of plant photosynthetic performance (Maxwell and Johnson, 2000) with optimal values of around 0.83 measured for most plant species. Values lower than 0.83 indicate a decreased function of PSII, and are thus seen for stressed plants (Björkman and Demmig, 1987). The decline might be due to an increase in protective non-radiative energy dissipation or photoinhibitory damage to the PSII reaction center (Maxwell and Johnson, 2000).
The chlorophyll fluorescence parameter Fv/Fm does not, however always give satisfactory results, because the Fv/Fm ratio is not always correlated with leaf chlorophyll content (Maxwell and Johnson, 2000). Strauss et al. (2006) demonstrated that drought stress conditions on barley varieties did not affect Fv/Fm. A similar effect was observed for dark chilling stress in soybean (Van Heerden et al., 2004). Furthermore, toxic cadmium did not cause a reduction in Fv/Fm when Phragmites australis was grown in presence of 50 or 100 µM cadmium (Pietrini et al., 2003).

1.6.2 Oxidative stress

Oxidative stress is related to the formation of reactive oxygen species (ROS). ROS is a collective term that includes all reactive forms of oxygen, including both the radical and non-radical species, that initiate and/or propagate radical chain reactions. These ROS are constantly produced endogenously as a result of normal plant metabolism (Foyer et al., 1994), but also can be produced as by-products of biotransformation reactions of toxins or xenobiotics (Polle, 2001). The action of ROS and the stress response are in a dynamic equilibrium between the rate of formation and further utilization so that the oxidative damage only takes place if the level of ROS exceeds the antioxidative capacity of the plant (Pflugmacher, 2004). ROS are thus produced in a higher amount if the plant is exposed to environmental stress conditions, and this enhanced amount of ROS needs to be scavenged in order to maintain the normal cellular functions and to prevent cellular damage (Gueta-Dahan et al., 1997).

To prevent cellular damage caused by ROS, cells have developed a protective system involving antioxidative enzymes, and free-radical traps (antioxidants) (Pflugmacher et al., 2006). Therefore, survival under stress conditions is only possible if several antioxidants act together with antioxidant enzymes in order to provide a good defense system and a quick and balanced regeneration of the active reduced forms of the antioxidants (Pflugmacher, 2004).
The marker enzymes of the antioxidative system are glutathione peroxidase (GPx: EC 1.11.1.9), glutathione reductase (GR: EC 1.6.4.2), glutathione S-transferase (GST: EC 2.5.1.18), peroxidase (POD: EC1.11.1.7), superoxide dismutase (SOD: EC 1.15.1.1), ascorbate peroxidase (APx: EC 1.11.1.11), and catalase (CAT: EC 1.11.1.6) (Fig. 1.6) (Pflugmacher, 2004). Antioxidants include glutathione, ascorbate, vitamins, and tocopherols (Asada, 1992; Polle, 2001; Pflugmacher et al., 2006). The intracellular glutathione (γ-glutamylcysteinylglycine), as the most abundant cellular thiol, is the most important component of the antioxidative defense system in plants (Alscher, 1989). GSH detoxifies ROS by acting as co-substrate for the biotransformation enzymes glutathione transferases and for the antioxidative enzyme glutathione peroxidase (Fig. 1.6) (Pflugmacher et al., 2001).

Glutathione S-transferases are a family of multifunctional, dimeric enzymes that catalyze the conjugation of GSH to compounds containing electrophilic centers. The primary functions of GSTs are the detoxification of endobiotic and xenobiotic compounds, as well as to play a role in providing antioxidative protection. In plants, GSTs have been found to play an important role in herbicide detoxification, and this mechanism has accounted for plant herbicide tolerance. The mechanism of action of this enzyme involves a system whereby GSTs transfer a glutathione-tag to a selected substrate and the resulting less reactive and more polar conjugate can then be actively transported out of the cytosol by a glutathione-conjugate transporter located in the tonoplast of the plant (Wagner and Mauch, 1997; Pflugmacher, 2004).

Superoxide dismutase is considered a key enzyme in the regulation of intracellular concentrations of superoxide radicals and peroxides. It dismutates two molecules of superoxide into oxygen and hydrogen peroxide: \( 2 \text{O}_2^- + 2 \text{H}^+ \xrightarrow{\text{SOD}} \text{O}_2 + \text{H}_2\text{O}_2 \). Hydrogen peroxide is then reduced by CAT, or GPx or APx. The CAT decomposes hydrogen peroxide to water and oxygen, whereas GPx and APx reduce hydrogen peroxide to water (Asada, 1992) (Fig. 1.6).
Figure 1.6: Schematic illustration of the antioxidative defence mechanism. ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; GPx, glutathione peroxidase; APx, Ascorbate peroxidase, MDHA, monodehydroascorbate; GSH, reduced glutathione; GSSG, oxidised glutathione, NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADP oxidised nicotinamide adenine dinucleotide phosphate.

GR plays a role in the control of endogenous hydrogen peroxide through an oxido-reduction cycle involving glutathione and ascorbate (Smith et al., 1989). It catalyzes the reduction of glutathione disulfide (GSSG) to GSH in an NADPH-dependent reaction (Fig. 1.6), and therefore plays an important role in protection against oxidative damage (Pflugmacher, 2004). The maintenance of the ratio between reduced GSH and GSSG is vital for the normal functioning of plant metabolism (Foyer et al., 1994). The change in ratio between reduced GSH and GSSG is therefore a clear sign of stress in the plants possibly affecting other metabolic pathways (Pflugmacher, 2004).

In plants, lipid peroxidation (LPO) may result from an increased amount of ROS due to exposure to cyanotoxins. The presence of antioxidants, such as ascorbate, GSH, and tocopherols helps in preventing lipid peroxidation (McDonald, 1999). Tocopherols are synthetised in plants and are important for membrane integrity as they scavenge lipid peroxy radicals thereby preventing the propagation of LPO in membranes (Nordberg and Arner, 2001). LPO induced by cyanobacterial toxins was detected in Medicago sativa by Pflugmacher et al. (2006).
BMAA was recently found to cause oxidative stress in the aquatic macrophyte *C. demersum* as it inhibits the action of antioxidant enzymes that scavenge ROS (Esterhuizen *et al.*, personal communication). Lobner and colleagues reported that one possibility for BMAA-induced oxidative stress is competition by BMAA with cystine at the cystine/glutamate antiporter leading to a decreased cystine uptake, and thus depletion of endogenous free radical scavenger glutathione, since cystine is the precursor for production of GSH (Lobner *et al.*, 2007; Liu *et al.*, 2009).

### 1.7 Scope and aims of the present study

Hazard characterization and risk assessment of cyanobacterial toxins in relation to human health requires the identification of exposure routes. The accumulation of BMAA in crop plants may provide a route for human intoxication as the BMAA can be carried through the food chain. Since BMAA has been found in cyanobacteria present throughout the world, this study may help to establish the existence of any risk to humans from the consumption of plants irrigated with water from dams with high cyanobacterial biomass and therefore high levels of BMAA. Based on this, the aims of this study were to:

1) investigate the effect of BMAA on the growth and development of *Nasturtium officinale* and *Daucus carota* by measuring the root, stem, leaf length, and total fresh weight,

2) analyze exposed plants for BMAA uptake using Liquid Chromatography Mass Spectrometry (LC-MS),

3) assess the effect of BMAA on photosynthetic performance of *N. officinale* from measurements of chlorophyll fluorescence yield following BMAA exposure through the growth medium,

4) evaluate the toxicological effects of BMAA on *N. officinale* by measuring the activity of oxidative stress responsive enzymes, and to

5) establish the existence of any risk to humans from the consumption of plants irrigated with water from dams with high cyanobacterial biomass and thus high BMAA levels.
2 MATERIALS AND METHODS

2.1 The effect of BMAA on growth and development of *N. officinale* and *D. carota*

*N. officinale* and *D. 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 liquify 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 was used (100, 300, 500 µgL⁻¹). The concentration of BMAA was set to a maximum of 500 µgL⁻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% H₂O₂ 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.
2.2 Effect of BMAA on *N. officinale* and *D. carota* seeds’ germination

The seeds were washed as above and were left in 4.2 and 42 μM BMAA solution overnight to germinate. For control plants, seeds were germinated in distilled water. The seeds were aseptically transferred onto media without BMAA in each growth container and grown as above. 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*.

2.3 Amino acid analysis using LC-MS for BMAA concentrations in *N. officinale* and *D. carota*

Seedlings (control and BMAA-treated) in replicates of three (with each replicate containing three plants) were washed in distilled water, methanol (98%), and again in distilled water to remove any surface associated BMAA. The plants were snap frozen in liquid nitrogen and lyophilized overnight in a Virtis bench top freeze drier (condenser temperature of -50°C and a vacuum of 245 mTorr). Samples were weighed and the weight of the pre-weighed Eppendorf tube was subtracted to determine the dry weight of the plants. Plant were homogenized in 0.1 M trichloroacetic acid (TCA) using an Elvehjem homogenizer (1000 μl of 0.1 TCA in each Eppendorf tube), vortexed vigorously and repeatedly, and allowed to stand for 30 minutes at room temperature. Samples were then sonicated in water bath for 3 hours, and replaced with ice cold water every hour. 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. It 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.
Samples were derivatized as described by Esterhuizen and Downing (2008) using the EZ:faastTM amino acid analysis kit for LC/MS (Phenomenex). BMAA was separated from other amino acids by liquid chromatography on a commercial column (Phenomenex AAA-MS 250 x 2.0 mm amino acid analysis column) using a Shimadzu UFLC liquid chromatography system coupled to a Shimadzu EV2010 MS. A solvent gradient was used with A: 10 mM ammonium formate in water and B: 10 mM ammonium formate in methanol (0.0 min = 68% B, 13.00 = 83% B, 13.01 = 68% B, 17.00 68% B) at a flow rate of 0.25 min and 1 µL sample injection volume. Column temperature was kept 35 ºC. The mass spectrometer ESI source (positive ion mode) temperature was set at 250 ºC. 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 ºC. 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.

2.4 BMAA ecotoxicology

2.4.1 Chlorophyll a fluorescence measurements

The control and BMAA-treated groups were grown as above for 10 days in order to get leaves big enough for analysis. All plants were dark adapted for 30 min before the measurements were started. Chlorophyll a fluorescence (expressed in relative units) was determined using a portable Handy Plant Efficiency Analyser (PEA, Hansatech, UK). Leaf fluorescence was determined after illumination with light intensity of 3000 µmolm$^{-2}$s$^{-1}$. The Fv/Fm values were obtained automatically using Handy PEA v 1.3 software.

2.4.2 Enzyme assays

The control and BMAA-treated groups were grown as above for seven days. Protein extraction was done as per Pflugmacher and Steinberg (1997) with slight modifications.
Control and BMAA-treated plants were separately ground to a fine powder in liquid nitrogen. The resulted powder was then 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), in relation 2:1 (v/w). Cell debris was removed by centrifuging the slurry at 10,000 g for 10 min (Optima™ L-80 XP Ultracentrifuge, Beckmann Coulter). The supernatant was 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 precipitation step which contained 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) and eluted in 3.5 ml of 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. The assay consisted of 1226 μL Bradford reagent, and 25 μL sample. Sodium phosphate buffer (20 mM, pH 7.0) was used for blank. Total protein content was determined in triplicate for each sample. Bovine serum albumin (BSA 98%, Sigma) was used to construct standard curve (Fig. 2.1).

![Standard curve used to quantify total protein from N. officinale extracts (R² = 0.992; y = 0.9301x; N = 3).](image-url)
Catalase (CAT) activity was determined by spectrophotometrical measurement according to Baudhuin et al. (1964), using H$_2$O$_2$ as substrate. Decrease of H$_2$O$_2$ was followed at 240 nm for three minutes with 30 sec intervals in sodium phosphate buffer (50 mM, pH 7.0). An Ultraspec 2100 pro UV / Visible Spectrophotometer (Amersham, Biosciences) was used. The assay consisted of 1250 μL sodium phosphate buffer (50 mM, pH 7.0), 100 μL H$_2$O$_2$ (100 mM), and 50 μL sample. The molar extinction coefficient (ε) for CAT is ε = 0.0361 (L.mmol$^{-1}$.cm$^{-1}$).

Glutathione reductase (GR) activity was assayed spectrophotometrically as described by Carlsberg and Mannervik (1985). The principle of this assay is that NADPH is oxidized to NADP$^+$, and this is accompanied by a decrease in absorbance that can be monitored at 340 nm. GSSG present in the reaction mixture is reduced to GSH by GR during the process: GSSG + NADPH + H$^+$ $\xrightarrow{GR}$ 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). The assay consisted of 850 μL sodium phosphate buffer (0.1 M, pH 7.5), 50 μL GSSG (20 mM), 50 μL NADPH (2 mM), and 50 μL sample. ε = 6.4 (L.mmol$^{-1}$.cm$^{-1}$).

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. Thus, the GR recycles the reduced form of GSH so that the total GSH remains essentially constant. 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), and gives an indication of the levels of GPx activity. The assay consisted of 800 μL sodium phosphate buffer (0.1 M, pH 7.5), 10 μL GR (2 U), 40 μL GSH (50 mM), 40 μL NADPH (2 mM), 10 μL H$_2$O$_2$ (9 mM), and 100 μL sample. ε = 6.4 (L.mmol$^{-1}$.cm$^{-1}$).
Superoxide dismutase (SOD) was measured spectrophotometrically using a commercial SOD kit (Sigma-Aldrich™). The SOD kit uses a highly water-soluble tetrazolium salt, WST [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]. SOD activity in the soluble fraction is measured using the colorimetric assay of xanthine/xanthine oxidase activity measured at 450 nm in a plate reader after incubation for 20 min. The WST produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of this reduction is linearly related to the xanthine oxidase activity, which is inhibited by SOD. The SOD activity in the samples is therefore calculated indirectly by its inhibition capacity. The assay consisted of 200 μL WST working solution, 20 μL enzyme working solution / dilution buffer, and 20 μL H₂O / sample.

Enzymatic activity was expressed in relation to the total protein content of the sample (Bradford, 1976). It is therefore reported in katal (kat) per milligram of protein (kat/mg 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 \( \Delta E = \) extinction change per minute, \( V = \) test volume (μL), \( \varepsilon = \) molar extinction coefficient (L.mmol\(^{-1}\).cm\(^{-1}\)), \( d = \) cuvette width, \( \Delta t = \) 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 accurate measurement requires at least four replicates for each sample.

Statistical analysis was performed using Statistica software (Statsoft, Inc. 2002). Analysis of variance (ANOVA) was carried out. The Tukey honest significant difference test was used to identify significant differences in the data sets (\( p < 0.05 \)).
3 RESULTS

3.1 The effect of BMAA on growth and development of *N. officinale* and *D. carota*

When the *N. officinale* seeds were grown in the absence of BMAA or in the presence of various concentrations of BMAA (100, 300, and 500 µg L\(^{-1}\)), the daily measurements of leaf, stem and root lengths as well as fresh weight revealed no difference in growth between control and BMAA-treated plants for the entire 7-day run as seen in the figure 3.1 (A-D). There was almost the same increase in length for both control and toxin-exposed plants throughout the study period.

When *D. carota* seeds were grown in absence or in presence of BMAA, results indicated that growth was not inhibited in presence of BMAA (100, 300, and 500 µg L\(^{-1}\)) at the concentration tested throughout the experiment. The leaf, shoot and root lengths as well as fresh weight in exposed plants did not differ significantly to control plants which were grown in medium lacking the BMAA (Fig. 3.2, A-D).

Fig. 3.3 (A-D) shows the results obtained when *N. officinale* were grown in medium without BMAA following overnight seeds germination in BMAA solution (4.2 and 42 µM). For control plants, seeds were germinated in distilled water. There was no difference in growth between control and BMAA-germinated *N. officinale* plants. When *D. carota* plants were grown in the same conditions, there was no difference between control and 4.2 µM BMAA-germinated plants (Fig. 3.4, A-D). However, for 42 µM BMAA-germinated plants, 40% of the *D. carota* roots fails to penetrate the growth medium, their hypocotyls (stems) were not elongated (straight) like control plants, and the growth of leaves was inhibited. The remaining 60% of *D. carota* roots were able to enter the growth medium, and there was no difference from control plants as it can be seen from Fig. 3.4 (A-D).
Figure 3.1: Daily measurements of *N. officinale* leaf (A), stem (B), and root (C) lengths as well as fresh weight (D) without BMAA (control, □) and in presence of 100 (●), 300 (■), and 500 µgL⁻¹ BMAA (■). Control plants did not receive BMAA. Averages represent triplicate growths as performed on three plants each.
Figure 3.2: Daily measurements of *D. carota* leaf (A), stem (B), and root (C) lengths as well as fresh weight (D) without BMAA (control, □) and in presence of 100 (■), 300 (■), and 500 µgL⁻¹ BMAA (■). Control plants did not receive BMAA. Averages represent triplicate growths as performed on three plants each.
Figure 3.3: Daily measurements of *N. officinale* leaf (A), stem (B), and root (C) lengths as well as fresh weight (D) when seeds were germinated in distilled water (control, □), in 4.2 (●) and 42 µM BMAA (■). Averages represent triplicate growths as performed on three plants each.
Figure 3.4: Daily measurements of *D. carota* leaf (A), stem (B), and root (C) lengths as well as fresh weight (D) when seeds were germinated in distilled water (control, □), in 4.2 (●) and 42 µM BMAA (■). Averages represent triplicate growths as performed on three plants each.
3.2 Bioaccumulation of BMAA in *N. officinale* and *D. carota*

The LC–MS system was validated using a range of dilutions of an authenticated BMAA standard (Sigma) against internal authenticated standards of homoarginine (HARG), methionine (Met), and homophenylalanine (HPHE) (Fig. 3.5).

**Figure 3.5:** Chromatogram of standard BMAA [Retention time (Rt): 9.1 min; m/z: 333] measured against an internal standard of homoarginine (Rt: 3.6 min; m/z: 317), methionine 03 (Rt: 8.1 min; m/z: 281), and homophenylalanine (Rt: 12.6 min; m/z: 308).
3.2.1 BMAA bioaccumulation in *N. officinale*

After BMAA extraction from *N. officinale*, it was derivatized, quantified by LC-MS, and expressed per dry weight of the sample. It was found that for all BMAA exposure concentrations tested, both total free and bound cellular BMAA increased as the concentration of BMAA in the growth medium increased. Total bound cellular BMAA concentration was higher than free cellular BMAA concentration for all BMAA exposure concentrations (Fig 3.6). 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 µgL−1 BMAA; but it decreased from day 3 to day 7 in plants treated with 300 µgL−1 BMAA. The total bound cellular BMAA decreased from day 3 to day 7 at 100 and 300 µgL−1; however, at 500 µgL−1, it increased from day 3 to day 5, and then decreased from day 5 to day 7 (Fig. 3.6). Control plants not exposed to BMAA had no free or bound cellular BMAA.

![Figure 3.6: 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 (■), 300 (■), and 500 µL−1 (■). Data are mean ± SD (n = 3). Control plants not exposed to BMAA had no free or bound cellular BMAA.](image-url)
3.2.2 BMAA bioaccumulation in *D. carota*

After BMAA extraction from carrot, derivatization, and quantification on a Shimadzu Liquid Chromatography Mass Spectrometer, it was noticed that both total free and bound cellular BMAA expressed per dry weight of the sample increased as the concentration of BMAA in the growth medium increased. Total bound cellular BMAA concentration was also higher than free cellular BMAA concentration for all BMAA exposure tested (Fig. 3.7). In addition, total free and bound cellular BMAA decreased from day five to day seven for all exposure concentrations (Fig. 3.7). Like for *N. officinale*, control plants not exposed to BMAA had no free or bound cellular BMAA.

![Graph showing BMAA uptake by *D. carota*](image)

**Figure 3.7:** 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 µgL⁻¹ (□); total cellular protein-associated BMAA obtained when the exposure concentration was 100 (■), and 500 µgL⁻¹ (□). Data are mean ± SD (n = 3). Control plants not exposed to BMAA had no free or bound cellular BMAA.

Typical chromatograms that reveal the absence of BMAA in control plants and the presence of BMAA in BMAA-treated plants are shown on the Fig. 3.8.
Figure 3.8: Chromatograms showing the absence of BMAA in *D. carota* grown in the medium without BMAA at day five (A), the presence of free cellular BMAA in *D. carota* (B) and in *N. officinale* (C) at day five at 500 µg.L\(^{-1}\), and the presence of bound cellular BMAA from *N. officinale* hydrolyzed sample from day five at 500 µg.L\(^{-1}\). For BMAA, Rt = 9.1 min, and m/z = 333. For tryptophan (Trp), Rt = 10.2 min; m/z: 333.

### 3.3 BMAA ecotoxicology

#### 3.3.1 Effect of BMAA on photosynthetic efficiency of *N. officinale*

Chlorophyll \(a\) fluorescence was used as a rapid diagnostic method to determine the health of *N. officinale* leaves when exposed to BMAA through the growth medium. The photosynthesis performance was determined after dark adaptation by analyzing Fv/Fm, which represents the maximum quantum yield of PSII (Maxwell and Johnson, 2000). Results showed that the Fv/Fm ratio values were 0.81 (± 0.02), 0.81 (± 0.02), 0.80 (± 0.03), and 0.78 (± 0.04) for control plants and for plants exposed to 100, 300, and 500.
µg.L\(^{-1}\) BMAA, respectively (n=10) (Fig. 3.9). However, this apparent trend was not statistically significant.

Figure 3.9: Clustered column of Fv/Fm ratio of dark adapted leaves according to BMAA concentration. Photosynthetic activity was assayed as Fv/Fm fluorescence. Data were carried out on \(N.\ officinale\) exposed to various (100, 300, and 500 µg.L\(^{-1}\)) concentration of BMAA for a period of 10 days. Data are mean ± SD (n = 10).

3.3.2 Effect of BMAA on oxidative stress response enzymes extracted from \(N.\ officinale\)

The selected antioxidant defence 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 (Fig 3.10 & 3.11).

There was a significant effect of BMAA concentration on CAT activity at the p<.05 level for the three concentrations tested [F(3, 26) = 9.26, p = 0.0002]. Post hoc comparisons using the Tukey HSD test indicated significant differences from controls (M = 84176.25,
SD = 11749.54) in all treated groups: 100 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).
Figure 3.10: 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).

There was no significant effect of any tested BMAA concentration on GR activity at the p<.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<.05 level for the three concentrations tested [F(3, 26) = 1.85, p = 0.162] (Fig. 3.10).

![Graph showing SOD activity](image)

Figure 3.11: 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).

There was a significant effect of BMAA concentration on SOD activity at the p<.05 level for the three concentrations tested [F(3, 32) = 12.46, p < 0.0001]. 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) (Fig 3.11). The increase in SOD activity was not significant at 500 µg.L⁻¹ (Fig. 3.11).
4 DISCUSSION

4.1 The effect of BMAA on growth and development of *N. officinale* and *D. carota*

Investigations into the effects of BMAA on the growth of *N. officinale* and *D. carota* demonstrated that BMAA had no effect on growth and development. After the exposure to the BMAA through the growth medium, BMAA-treated plants looked like control plants throughout the study period. Studies undertaken by Esterhuizen *et al.* (2009, personal communication) showed no difference in growth between control and BMAA-treated *C. demersum* plants for a period of 24 hrs. Prolonged exposure to BMAA or high BMAA concentration may have an impact on plant development. Brenner *et al.* (2000) showed that when *Arabidopsis* seedlings were transferred to medium containing 50 μM BMAA or greater, both primary root growth and shoot growth were severely inhibited due to blockage of transduction of light signals by BMAA. However, the concentrations used by Brenner *et al.* (2000) are higher and do not reflect the BMAA concentrations found in the environment (based on extrapolation from studies by Cox *et al.*, 2005; Esterhuizen and Downing, 2008).

BMAA concentration, as high as 42 μM, had no adverse effect on the germination of *N. officinale*. However, the germination of *D. carota* seeds was affected by BMAA at this concentration since 40% of the roots failed to penetrate the growth medium. As a result, the hypocotyls became curved, and the growth of leaves was inhibited. BMAA was also found to have an inhibitory effect on *Arabidopsis* root growth at 50 μM by blocking plant *Arabidopsis* glutamate receptor-like (AtGLR) signalling (Brenner *et al.*, 2000). Other cyanotoxins like microcystins were also inhibitors of growth and development in potato shoots and mustard seedlings under laboratory conditions due to necrosis of tissues, which was accompanied by a reduction in chlorophyll content (McElhiney *et al.*, 2001).
4.2 Bioaccumulation of BMAA in *N. officinale* and *D. carota*

BMAA was found to bioaccumulate both in *N. officinale* and *D. carota* following BMAA exposure through the growth medium. The total amounts of BMAA detected in plants extracts increased as the concentrations of BMAA in the growth medium increased. The same results were found for the aquatic macrophyte *C. demersum* by Esterhuizen *et al.* (personal communication). Both free and bound cellular BMAA was detected in *N. officinale* and *D. carota*. BMAA can bind to or incorporated into proteins (Murch *et al.*, 2004), as do many other non-protein amino acids (Neilan *et al.*, 1999). BMAA has been artificially incorporated into synthetic peptides (Seebach *et al.*, 1994). BMAA uptake in *N. officinale* increased from day 3 to day 5, and then decreased from day 7. This may have occurred as a result of the metabolism of BMAA by plants exposed to the toxin for longer periods since amino acids are used by the plants as nitrogen source (Syennerstam *et al.*, 2007).

Protein-bound BMAA in most instances occurred at higher concentrations than free cellular BMAA (Fig. 3.6 & 3.7). The ratio between the protein-bound and free BMAA is between 1:1 and 8:1 for *N. officinale*, and 1:1 and 2:1 in *D. carota*. The bound cellular BMAA also occurred in higher amounts than free cellular BMAA in cycad seeds (Murch *et al.*, 2004), and in free living cyanobacteria (Cox *et al.*, 2005; Esterhuizen and Downing, 2008). However in *C. demersum*, free cellular BMAA was higher than bound cellular BMAA and this was attributed to short exposure time (Esterhuizen *et al.*, personal communication). The decrease in bound/free ratio from *N. officinale* to *D. carota* might be due to slow germination and or slow growth of *D. carota* compared to *N. officinale* since measurable *N. officinale* leaf, stem and root tissue was obtained from day 3, whereas adequate biomass for analysis of *D. carota* was only obtained from day 5.
4.3 BMAA ecotoxicology

4.3.1 Effect of BMAA on photosynthetic efficiency of *N. officinale*

The values of Fv/Fm ratio usually reflect the potential quantum efficiency of PSII, and are thus used as a sensitive indicator of photosynthetic performance (Maxwell and Johnson, 2000). For most plant species, the optimal Fv/Fm values are around 0.83; therefore, leaf values lower than this are generally seen when the plant has been exposed to stress (Björkman and Demmig, 1987). Maxwell and Johnson (2000) reported that the decrease in Fv/Fm ratio might be due to an increase in protective non-radiative energy dissipation or to photoinhibitory damage to the PSII reaction centre. Results showed that the Fv/Fm ratio values were 0.81 (± 0.02), 0.81 (± 0.02), 0.80 (± 0.03), and 0.78 (± 0.04) for control plants and for plants exposed to 100, 300, and 500 µg.L⁻¹, respectively (n = 10). These values may suggest that the photosynthetic apparatus of *N. officinale* was not damaged since no statistical difference was observed between control and BMAA-treated plants. The changes observed in *N. officinale* suggest the possibility of bioassay or biomonitoring since BMAA is an active toxic substance that can cause some changes in the plant. No significant difference in Fv/Fm ratio was also observed when broccoli and mustard plants were grown in water or mineral medium supplemented with a purified MC-cyanobacteria extract, 0.1-10 µg equiv. MC-LR/L (Järvenpää et al., 2007). Strauss *et al.* (2006) demonstrated that drought stress conditions on barley varieties did not affect Fv/Fm. A similar effect was observed for dark chilling stress in soybean (Van Heerden *et al.*, 2004). Furthermore, toxic cadmium did not cause a reduction in Fv/Fm when *Phragmites australis* was grown in presence of 50 or 100 µM cadmium (Petrini *et al.*, 2003). Chlorophyll fluorescence parameters (like Fv/Fm) do not always give satisfactory results, because the Fv/Fm ratio, which represents the PSII functional status, is not always correlated with leaf chlorophyll content (Maxwell and Johnson, 2000).
4.3.2 Effect of BMAA on oxidative stress response enzymes in *N. officinale*

In this study, CAT, GR, GPx, and SOD were used as markers for oxidative stress. BMAA was found to have an inhibitory effect on the activity of some of these enzymes. The inhibition was not significant for GR and GPx for the three concentrations tested. For CAT, the inhibition was significant for the three concentrations tested. The inhibition effect was concentration dependent since the inhibition was more pronounced as the BMAA exposure concentration increased. SOD activity for plants exposed to 100 µg.L$^{-1}$ was not significantly different than the control plant. However, the activity in plants exposed to 300 µg.L$^{-1}$ was significantly different to controls. The only exception to trends in inhibition was for 500 µg.L$^{-1}$ exposure concentration for SOD assay, where the enzyme activity for this concentration was higher than control (Fig. 3.11). However, the increase in SOD activity was not significant at 500 µg.L$^{-1}$. Esterhuizen *et al.* (personal communication) suggested that the inhibition of oxidative stress-response enzymes might be due to a direct BMAA interaction with the enzyme resulting in a conformational change in the protein leading to biological activity loss.

Since BMAA inhibits the activity of the antioxidant defence enzymes in *N. officinale*, it indirectly causes oxidative stress. BMAA induces oxidative stress and changes protein function via incorporation of BMAA into proteins (Murch *et al.*, 2004). BMAA was recently found to cause oxidative stress in the aquatic macrophyte *C. demersum* as it inhibits strongly the action of antioxidant enzymes (CAT, GR, POD, SOD, GPx, and GST) that scavenge ROS (Esterhuizen *et al.*, personal communication). Lobner and colleagues reported that one possibility for BMAA-induced oxidative stress is competition by BMAA with cystine at the cystine/glutamate antiporter leading to a decreased cystine uptake, and thus depletion of endogenous free radical scavenger glutathione, since cystine is the precursor for production of GSH (Lobner *et al.*, 2007; Liu *et al.*, 2009). Other cyanotoxins like MC-LR were also found to enhance the anti-
oxidative enzyme activities of POD, SOD, GPx, and APx, following toxin exposure in *C. demersum*, indicating ROS formation and on-going ROS detoxication in the plant (Pflugmacher, 2004).

4.4 Correlation between BMAA bioaccumulation and BMAA ecotoxicology in *N. officinale*

BMAA bioaccumulation and BMAA ecotoxicity were found both to depend on BMAA concentration exposure in *N. officinale*. Indeed, the total amounts of BMAA detected in plants extracts increased as the concentrations of BMAA in the growth medium increased, and inhibition of oxidative stress-response enzymes was more pronounced as the BMAA exposure concentration increased (Fig. 3.6 & 3.10). No significant linear relationship was observed, presumably due to saturation at concentrations below 500 µg.L\(^{-1}\). Therefore, the inhibition of antioxidant defence enzymes by BMAA correlated with the BMAA bioaccumulation in *N. officinale* since the activity of oxidative stress response enzymes decreased as the total cellular free BMAA (Fig. 4.1) or total cellular protein-associated BMAA (Fig. 4.2) expressed per dry weight of the sample increased. No significant linear relationship was however observed. The same correlation was also found for the aquatic macrophyte *C. demersum*. Esterhuizen *et al.* (personal communication) suggested that the inhibition of oxidative stress-response enzymes might be due to a direct BMAA interaction with the enzyme resulting in a conformational change in the protein leading to biological activity loss.
Figure 4.1: A plot of CAT activity [µkat mg\(^{-1}\) protein] (A), GR activity [nkat mg\(^{-1}\) protein] (B), and GPx activity [nkat mg\(^{-1}\) protein] (C) against total cellular free BMAA expressed per dry weight of the sample, extracted from \(N.\ officinale\) after 7 days, following exposure to varying BMAA concentrations (0, 100, 300, and 500 µgL\(^{-1}\)).
Figure 4.2: A plot of CAT activity [µkat mg⁻¹ protein] (A), GR activity [nkat mg⁻¹ protein] (B), and GPx activity [nkat mg⁻¹ protein] (C) against total cellular bound BMAA expressed per dry weight of the sample, extracted from *N. officinale* after 7 days, following exposure to varying BMAA concentrations (0, 100, 300, and 500 µg L⁻¹).
5 CONCLUSION

BMAA had no effect on growth and development of *N. officinale* and *D. carota*. After the exposure to the BMAA through the growth medium, BMAA-treated plants looked like control plants. However, for 42 µM BMAA-germinated plants, 40% of the *D. carota* roots fails to penetrate the growth medium, their hypocotyls were not elongated like control plants, and the growth of leaves was inhibited. The uptake and bioaccumulation of BMAA was confirmed in *N. officinale* and *D. carota*, and was found to be concentration-dependent. Both free and bound cellular BMAA was detected following BMAA exposure through the growth medium. The inhibition of antioxidant enzymes upon exposure of *N. officinale* to BMAA through the growth medium was also shown. The inhibition of antioxidant defence enzymes by BMAA followed the trend of BMAA bioaccumulation in *N. officinale*. The photosynthetic apparatus of *N. officinale* was not significantly damaged. The changes observed in *N. officinale* suggest the possibility of bioassay or biomonitoring since BMAA is an active toxic substance that can cause some changes in the plant. The possible mechanism for human exposure to large amounts appears to be BMAA biomagnification through the food chain. The uptake and accumulation of BMAA in edible terrestrial plants may constitute another route of human exposure to BMAA; it may now be prudent to avoid spray irrigation of edible plants with waters from dams with high cyanobacterial biomass and therefore high BMAA levels. Identifying and avoiding all sources of BMAA will be a great achievement since BMAA is highly toxic substance. Further investigations are needed to analyze the uptake, accumulation, and ecotoxicology of BMAA in other crop plants, and to examine the fate of BMAA in these plants particularly its distribution and metabolism.
6 REFERENCES


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