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β-N-Methylamino-L-alanine (BMAA) uptake by the aquatic macrophyte *Ceratophyllum demersum*

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ABSTRACT

Free-living freshwater cyanobacteria contain BMAA in both free cellular and protein-associated forms. Free BMAA released on bloom collapse or during cellular turnover creates a potential source of the non-proteinogenic amino acid for bioaccumulation and biomagnification in aquatic ecosystems. Uptake of free amino acids is well documented in macrophytes and the potential for aquatic macrophytes to bioaccumulate BMAA therefore poses a potential threat where such macrophytes constitute a food source in an ecosystem. BMAA uptake and accumulation by the aquatic macrophyte *Ceratophyllum demersum* was therefore investigated. Rapid uptake of significant amounts of BMAA was observed in *C. demersum*. Both free and protein-associated BMAA were observed with protein association following accumulation of free BMAA. The protein association suggests potential biomaccumulation by aquatic macrophytes and offers a possibility of phytoremediation for BMAA removal.

1. Introduction

Increased demand for surface water, as well as industrial and agricultural pressure on water resources, has resulted in eutrophication and an increased frequency of cyanobacterial blooms (Sivonen and Jones, 1993). Cox et al. (2005), Banack et al. (2007) and Esterhuizen and Downing (2008) reported BMAA in varying amounts in free-living cyanobacteria. Upon bloom senescence, free and protein-associated BMAA may be released into the surrounding environment, making it available for uptake or absorption by other organisms.

The high incidence of ALS/PDC in Guam was associated with plant bioaccumulated and animal biomagnified BMAA from cyanobacterial symbiotic *Nostoc* (Murch et al., 2004b). The existence of Canadian ALS cases, where BMAA was found in the brain tissue (Cox et al., 2003), suggests alternative human exposure routes. Since the known source of environmental BMAA is cyanobacteria (Cox et al., 2003, 2005; Murch et al., 2004a; Banack et al., 2007; Esterhuizen and Downing, 2008; Metcalf et al., 2008) and free-living cyanobacteria offer a potential source for BMAA exposure, it seems plausible that alternative routes of exposure may include bioaccumualtion of BMAA by plants and subsequent biomagnification by grazing. BMAA is a neurotoxin as it acts as a glutamate agonist (Lobner et al., 2007; Rao et al., 2006) in its carbonate form resulting in motor neuron selective toxicity via an excitotoxic mechanism (Nunn and O'Brien, 1989).

Uptake of amino acids from their surrounding environment by plants (McDaniel et al., 1982) and mis-incorporation of non-protein amino acids (Rodgers and Shiozawa, 2008), presumably as a defense mechanism based on toxicity to grazers (Crine and Lemieux, 1982; Rosenthal, 2001), have been well documented. The production of BMAA by cyanobacteria may therefore also constitute a defense mechanism if significant ecotoxicological effects can be demonstrated. Irrespective of possible function, BMAA released by cyanobacteria may be bioaccumulated and biomagnified in an aquatic system if taken up as demonstrated in the terrestrial food chain in Guam (Murch et al., 2004a). Neilan et al. (1999) showed non-protein amino acid incorporation into cyanobacterial proteins. The incorporation of BMAA into proteins may therefore be expected. BMAA has also been artificially incorporated into synthetic peptides by Seebach et al. (1994).

Plants are primary producers in all food chains and are therefore an essential indicator species for toxin bioaccumulation. BMAA uptake by aquatic plants may also constitute the first bioaccumulation and biomagnification link in an aquatic food chain. Three amino acid uptake systems (neutral, acidic and basic) have been characterised in plant cells. The three systems have a monovalent positively charged amino acid–proton carrier complex in common at the transport site (Wyse and Komor, 1984). BMAA is a neutral non-proteinogenic amino acid and is conceivably taken up by the conventional basic proteinogenic amino acid uptake system. We therefore investigated BMAA uptake by the aquatic macrophyte *Ceratophyllum demersum* and subsequent

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accumulation and distribution between free and protein-associated BMAA in *C. demersum*.

2. Methods and materials

The aquatic macrophyte *C. demersum* was selected for exposure as it occurs widely in nutrient-rich freshwater in tropic and temperate regions and as a submerged macrophyte would have maximum exposure to BMAA. *C. demersum* was purchased from Aqua Global (Dr. Jander & Co. OHG, Seefeld, Germany) and cultured for 3 days in a synthetic medium (Provasoli) mimicking its natural environment (Pflugmacher et al., 2006). The medium acts as a buffer and supplies essential nutrients needed for growth. The synthetic medium consists of de-ionized water containing $CaCl_2$ [0.2 g L^{-1}], $NaHCO_3$ [0.103 g L^{-1}] and sea salt [0.1 g L^{-1}] (aquarium water). Plants were maintained at a constant temperature of 23 °C (±1 °C), irradiance of 12 μE m^{-2} s^{-1} provided by plant growth lamps (HQL) and a photoperiod of 14:10 hours. All chemicals used were purchased from Sigma Aldrich A.R.

2.1. BMAA exposure

Filter sterilized aquarium water (0.2 μm, GH Polypro from Pall Corporation) containing BMAA (Sigma–Aldrich, L-BMAA hydrochloride B-107) at 0.5, 1, 5, 50 or 100 μg L^{-1} were prepared as exposure media. The levels selected reflect the potential free BMAA levels released upon bloom senescence based on published intracellular unbound BMAA amounts per gram biomass for a range of cyanobacteria (Cox et al., 2005; Banack et al., 2007; Esterhuizen and Downing, 2008). *C. demersum* sections (approximately 3 cm in length) were washed 4 times with distilled water to remove contaminants prior to exposure to BMAA (in quintuplicate) for 24 h, being the shortest time period over which sufficient BMAA was taken up based on trial experiments. Replicates were incubated separately, each in 100 ml of exposure media. Controls consisted of BMAA in aquarium water omitting *C. demersum* as well as *C. demersum* in aquarium water omitting BMAA, to ensure that no endogenous BMAA was present and to investigate the degradation/disappearance of BMAA, respectively. *C. demersum* sections were removed from the exposure media after 24 h and washed 3 times each with water and methanol to remove any molecules that may have adhered to the plant surface. Sections were then snap-frozen with liquid nitrogen and stored at −80 °C.

C. demersum sections were also exposed to 100 μg L^{-1} BMAA in quintuplicate for varying time periods (0.5, 1, 2, 4, 6 and 24 h). The BMAA exposure stock was prepared using BMAA (Sigma) and sterilized aquarium water. Negative controls were prepared using *C. demersum* sections in aquarium water without BMAA. After each of the exposure times, the *C. demersum* sections were removed and washed 4 times each with water and methanol, snap-frozen with liquid nitrogen and stored at −80 °C.

2.2. BMAA analysis

C. demersum sections were ground to a fine powder in liquid nitrogen and lyophilized overnight using a Gamma 1–20 freeze-drier from Christ (condenser temperature of −20 °C and vacuum of 300 m Torr). The dry weight of the powder was determined and resuspended in 0.5 ml 0.1 M trichloroacetic acid (TCA) by repeated vortexing. Following an incubation of 30 min at room temperature the supernatant, containing free BMAA, was collected by centrifugation (Eppendorf MiniSpin bench top centrifuge at 10,000*g* for 10 min at room temperature). The resultant pellet was washed with a further 0.5 ml 0.1 M TCA, which was then added to the original supernatant containing the free BMAA. Protein-associated BMAA was released from the pellet by liquid acid hydrolysis in 6 M HCl and 2% thioglycolic acid at 110 °C for 24 h in an inert atmosphere. Hydrolyzed extracts were filtered through a 0.22 μm filter (cellulose acetate) and the pH was correct to between 1 and 2 with NaOH before derivitization.

Samples were derivitized as described by Esterhuizen and Downing (2008) using the EZ:faast™ amino acid analysis kit for LC/MS (Phenomenex). BMAA was separated from other amino acids by liquid chromatography on a commercial column (Phenomenex AAA-MS 250 mm × 2.0 mm amino acid analysis column) using an Agilent Technologies 1200 liquid chromatography system coupled to an Applied Biosystems 3200 Q trap MS/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 constant at 35 °C.

The mass spectrometer ESI source (positive ion mode) temperature was set at 425 °C. The ion scan range was between 100 and 600 m/z. Ionspray voltage was set at 3000 V with a declustering potential of 20 V and an entrance potential of 10 V. Data were analyzed using Analyst 1.4.2 software. The liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) 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.

Statistical analysis was performed using Statistica software (Statsoft, Inc. 2002). ANOVA variance of data was carried out followed by the Newman–Keuls post hoc test and the Tukey honest significant difference test to identify significant differences in the data sets ($p < 0.05$). Uptake rates were calculated over the entire 24 h period, being the period over which preliminary experiments showed sufficient uptake to determine rates.

3. Results

C. demersum controls not exposed to BMAA contained no free cellular or protein-associated BMAA after a period of 24 h. Positive controls, containing no plant sections, showed a 5% (±1%) reduction in BMAA after 24 h. At all exposure concentrations significant amounts of BMAA were taken up by *C. demersum* (Figs. 1 and 2). Total BMAA in the plant section at 24 h was linearly related to the exposure concentrations ($R^2 = 0.9428$, $p = 0.0059$) over the exposure concentration range. For concentrations up to 50 μg L^{-1}, the correlation was stronger ($R^2 = 0.9755$, $p = 0.0129$) suggesting a saturation for uptake of below 100 μg L^{-1}. Protein-associated BMAA constituted between 3% and 13% of the total BMAA in the plant sections.

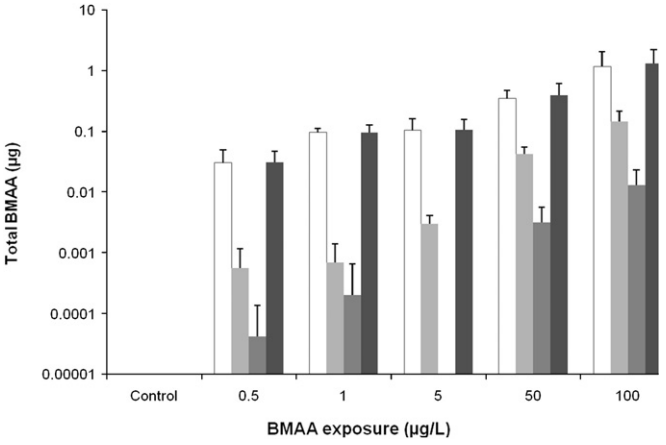

Fig. 1. BMAA uptake by *C. demersum* after 24 h exposure. $N = 5$, error bars denote standard deviation. □ Total BMAA remaining in exposure media, ▒ total cellular free BMAA extracted from *C. demersum*, ■ total cellular protein-associated BMAA, ■ total BMAA after 24 h.

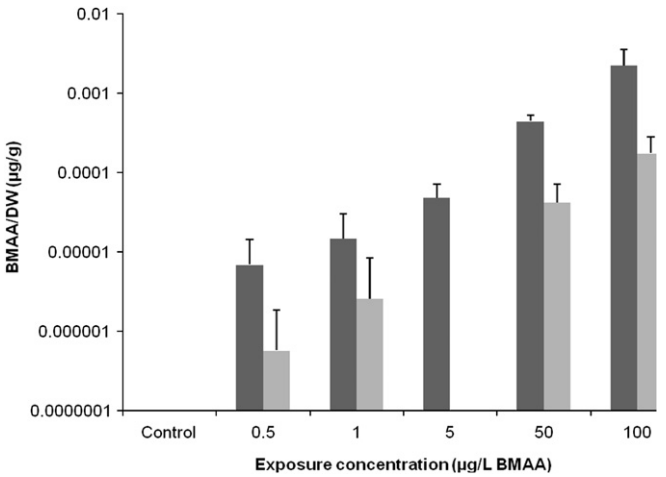

Fig. 2. BMAA (Sigma) uptake by *C. demersum* after 24 h exposure expressed per dry weight of the sample. $N = 5$, error bars denote standard deviation. ■ Total free cellular BMAA per dry weight after 24 h, ▒ total protein-associated cellular BMAA per dry weight after 24 h.

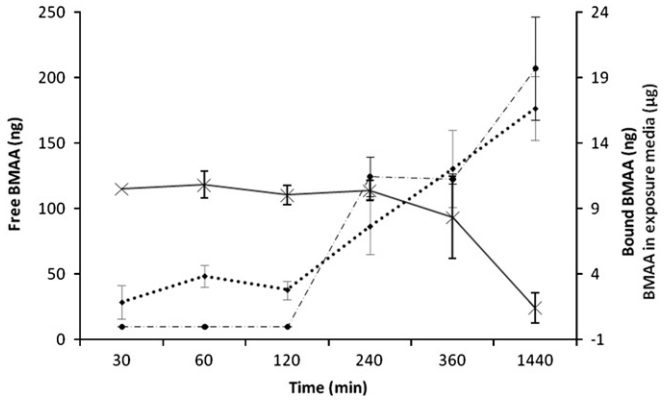

Fig. 3. Total BMAA remaining in exposure media of 100 ug L^{-1}. Total free cellular BMAA and protein-associated BMAA after uptake by *C. demersum* over a period of 24 h. $N=5$, error bars denote standard deviation. —×— BMAA in exposure media, —◆— cellular free BMAA in *C. demersum*, ——●—— cellular bound BMAA over a period of 24 h.

BMAA removal from the media corresponded to the appearance of BMAA in both free and bound forms in the plant sections. The rate of disappearance of BMAA from the medium (0.068 µg min^{-1}) was markedly higher than the loss in the controls lacking plants (0.27 ng min^{-1}) indicating binding to or uptake by the plant. The rate of BMAA loss from the medium also corresponded to the rate of appearance (0.8945 ng min^{-1} unbound and 0.0045 ng min^{-1} bound) of BMAA in the plant section (Fig. 3). The highest rates of BMAA removal from the medium and increase in BMAA in the plants occurred after 120 min.

4. Discussion

BMAA uptake by *C. demersum* is proportional to BMAA exposure concentration. The combined amount of BMAA retrieved from the plant material, the remaining BMAA in the exposure media and the BMAA lost to degradation (as determined by control experiment) was less than the initial amount of BMAA in the exposure media. This may be explained by the loss of BMAA due to washing of the plant with water and methanol, which may have resulted in the removal of bound BMAA from the plant exterior. Plants use amino acids as a source of nitrogen (Svennerstam et al., 2007); however, the mechanism by which the plant access this nitrogen is unknown. The decrease in total BMAA after 24 h may be attributed to break down of BMAA and subsequent nitrogen utilization. Fig. 3 shows a 2 h delay in BMAA protein association with an accumulation rate of 0.0045 ng min^{-1}. The ratio of free to bound BMAA was 13:1 for concentrations 0.5–50 µg L^{-1}; however, the ratio increased to 89:1 at 100 µg L^{-1} BMAA exposure concentration. The increased protein association rate at increased uptake rate suggests possible metabolism by the plant at low BMAA concentrations.

BMAA uptake by *C. demersum* follows Michaelis–Menten kinetics with a tendency to saturation. This corresponds with the findings of Borstlap et al. (1986) for amino acid uptake by plant cells.

Much lower BMAA amounts were attained than previously reported in cycads (Banack and Cox, 2003a, b; Murch et al., 2004a). Longer duration of exposure may result in a higher uptake of BMAA as well as a changed ratio of free and bound BMAAs.

These results constitute the first report of bioaccumulation of BMAA by an aquatic macrophyte. The use of environmentally relevant exposure concentrations (based on Cox et al., 2005; Esterhuizen and Downing, 2008) indicate that BMAA released from cyanobacterial blooms may be bioaccumulated in aquatic biota and potential grazers. Such bioaccumulation and subsequent grazing allows the possibility of biomagnification in aquatic systems as demonstrated for a terrestrial system by Murch et al. (2004a, b). Additionally, the rapid uptake by, and protein association in *C. demersum* raises the question of bioaccumulation in terrestrial plants irrigated with bloom containing waters.

Unlike the ratio of free to protein-associated BMAA in cycad seeds (Murch et al. 2004a) or in free-living cyanobacteria (Cox et al., 2005; Esterhuizen and Downing, 2008), protein-associated BMAA in *C. demersum* was lower than the free BMAA. This was attributed to the relatively short exposure time.

5. Conclusion

This is the first report of uptake and accumulation of BMAA by an aquatic plant. The only currently known possible human exposure routes to BMAA are via the terrestrial system on Guam (via cycad and fruit bat) or by direct ingestion of cyanobacteria.

In conclusion, the rapid uptake and protein association of BMAA by the aquatic macrophyte, *C. demersum*, indicates a possible route for biomagnification and exposure to BMAA originating from free-living cyanobacteria. Additionally, the possibility of exposure via terrestrial plants as a result of irrigation with water containing BMAA is of potential concern. Finally, the potential for phytoremediation of water containing BMAA should not be overlooked.

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