Ecotoxicology and Environmental Safety 208 (2021) 111515

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Research paper

Toxicity and bioaccumulation of two non-protein amino acids synthesised by cyanobacteria, β-*N*-Methylamino-L-alanine (BMAA) and 2,4-diamino-butyric acid (DAB), on a crop plant

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ARTICLE INFO

Edited by Dr G. Liu

Keywords:
BMAA
DAB
Non-protein amino acid
Cyanobacteria

ABSTRACT

In order to study the toxicity of the cyanobacterial non-protein amino acids (NPAAs) L-β-*N*-methylamino-L-alanine (BMAA) and its structural isomer L-2,4-diaminobutyric acid (DAB) in the forage crop plant alfalfa (*Medicago sativa*), seedlings were exposed to NPAA-containing media for four days. Root growth was significantly inhibited by both treatments. The content of derivatised free and protein-bound BMAA and DAB in seedlings was then analysed by LC-MS/MS. Both NPAAs were detected in free and protein-bound fractions with higher levels detected in free fractions. Compared to shoots, there was approximately tenfold more BMAA and DAB in alfalfa roots. These results suggest that NPAAs might be taken up into crop plants from contaminated irrigation water and enter the food chain. This may present an exposure pathway for NPAAs in humans.

1. Introduction

An emerging group of toxins known as non-protein amino acids (NPAAs) has recently been detected worldwide (Jiao et al., 2014; Li et al., 2018; Merel et al., 2013; Metcalf et al., 2015; Réveillon et al., 2014; Richer et al., 2015; Violi et al., 2019b). NPAAs are naturally-occurring amino acids that are not genetically coded for protein synthesis (Bell, 2003; Rodgers and Shiozawa, 2008). Some NPAAs, such as citrulline and ornithine, are common metabolic intermediates in plants and animals. Furthermore, NPAAs that are structural analogues of protein amino acids may be synthesised by plants to disable predators (Huang et al., 2011; Janzen et al., 1977; Rodgers et al., 2018) and as allelochemicals (Bertin et al., 2007; Hachinohe and Matsumoto, 2007; Rosenthal, 2001). In some cases, NPAA toxicity is due to their ability to replace protein amino acids in protein synthesis through amino acid 'mimicry' (Song et al., 2017), thus becoming mistakenly incorporated into proteins in place of a protein amino acid (Fowden, 1981; Rosenthal, 2001). This study is focused on the NPAA L-β-*N*-methylamino-L-alanine (BMAA) and its structural isomer, L-2,4-diaminobutyric acid (DAB). Another isomer of BMAA, N-(2-aminoethyl)glycine (AEG), exists in nature (Réveillon et al., 2014; Violi et al., 2019a, 2019b). However, studies suggest AEG is the least toxic of the three isomers (Main and Rodgers, 2018; Metcalf et al., 2015) and, therefore, was not investigated in the present study.

BMAA was first discovered when researchers were investigating the cause of the high incidence of a neurological disorder, known as amyotrophic lateral sclerosis-parkinsonism dementia-complex (ALS-PDC), on the West Pacific island of Guam in the 1950s. BMAA was detected in flour from cycad seeds (*Cycas circinalis*), where it appeared to be derived from cyanobacteria of the *Nostoc* genus living symbiotically in the roots of the cycad plant (Cox et al., 2003). It was found to bioaccumulate in the food chain via a pathway where the cycad seeds were eaten by fruit bats, which were then consumed by the indigenous Chamorro people on Guam (Banack et al., 2006; Cox et al., 2003).

BMAA is toxic to both animals and plants. In animals, the most prominent symptoms involve neurotoxicity (Cox et al., 2016; Muñoz-Sáez et al., 2015; Tan et al., 2018), but it has also been shown to have cytotoxic and genotoxic effects (Lepoutre et al., 2018) and to induce oxidative stress (Liu et al., 2009; Lobner et al., 2007). DAB is a toxic compound found in the flatpea, *Lathyrus sylvestris*, and also in the seeds of many other plants (Nigam and Ressler, 1966; Ressler et al., 1961). DAB also exhibits neurotoxicity (Chen et al., 1972; Chiu et al., 2013; Main and Rodgers, 2018; Weiss et al., 1989). In plants, BMAA toxicity has been shown in aquatic plants (macrophytes) where oxidative stress

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https://doi.org/10.1016/j.ecoenv.2020.111515
Received 27 May 2020; Received in revised form 2 October 2020; Accepted 13 October 2020
Available online 21 October 2020

has been implicated as a potential mechanism. In *Ceratophyllum demersum*, BMAA exposure had an inhibitory effect on antioxidant enzymes such as catalase, glutathione peroxidase, superoxide dismutase and guaiacol peroxidase (Esterhuizen-Londt et al., 2011b). Additionally, activity of the antioxidant enzyme peroxidase significantly increased in four aquatic plant species following 7-day treatment with BMAA, however, in three of the species, a return to baseline was observed after 14-days. Furthermore, higher concentrations of BMAA led to decreased peroxidase activity in the three bryophyte species (Contardo-Jara et al., 2013). BMAA also caused hypocotyl elongation and inhibited cotyledon unfolding in light-grown *Arabidopsis* (Brenner et al., 2003). DAB at high concentrations (2 mM) inhibited pollen tube germination and growth in *Lathyrus sylvestris*, *Vicia unijuga*, *Pisum sativum* and *Cicer arietinum* (Simola, 1967). DAB was also found to inhibit seed germination and growth in lettuce (*Lactuca sativa*) at concentrations between 1 and 10 mM (Wilson and Bell, 1979). It also showed toxicity (EC50 between 25 and 50 μM) in a *Lemna* growth assay (Gulati et al., 1981).

In view of the toxicity of BMAA and DAB, and the possibility that BMAA may be involved in the development of ALS-PDC, there is concern that humans may be exposed to these toxins (Brand, 2009; Lance et al., 2018). Knowledge of the distribution of these NPAAs in the environment will assist in assessing the likelihood of exposure.

BMAA is produced by all five known morphological groups of cyanobacteria (Cox et al., 2005) and has been detected in several species of dinoflagellates and diatoms (Jiang et al., 2014; Lage et al., 2014; Main et al., 2018; Réveillon et al., 2016; Violi et al., 2019a, 2019b). DAB is also produced by many cyanobacteria, often in conjunction with BMAA, but sometimes alone (Al-Sammak et al., 2014; Krüger et al., 2010; Violi et al., 2019a, 2019b). Cox et al. (2003) detected BMAA in plant species such as the water fern (*Azolla filiculoides*) and the angiosperm *Gunnera kauaiensis*; they assumed it was related to the cyanobacterial symbionts present in these species (Cox et al., 2003). Similarly, BMAA was detected in the aquatic macrophyte *Hydrilla* when it was associated with epiphytic cyanobacteria, but not detected in epiphyte-free *Hydrilla* (Bidigare et al., 2009). However, there have been reports of BMAA production in cyanobacteria-free cycad tissue (Marler et al., 2010). The widespread occurrence in cyanobacteria has led to the suggestion that ingestion or inhalation may be a potential route of toxin exposure for humans (Mello et al., 2018). Inhalation as a route of cyanotoxin exposure is supported by studies showing that humans routinely inhale aerosolised cyanobacteria, which can be harboured in the nostrils and lungs (Facciponte et al., 2018).

BMAA has been shown to be taken up from the environment in several plants including the aquatic plants *Lomariopsis lineata, Fontinalis antipyretica, Riccia fluitans* and *Taxiphyllum barbieri* (Contardo-Jara et al., 2013), watercress (*Nasturtium officinale*) and wild carrot (*Daucus carota*) (Niyonzima, 2010), wheat (*Triticum aestivum*) (Contardo-Jara, 2014, 2018) and Chinese cabbage (Li et al., 2019), while DAB accumulation has not yet been examined. The evidence of bioaccumulation of BMAA and co-occurrence of the two toxins suggests other terrestrial plants may be susceptible to bioaccumulation and serve as a route of human exposure. Another concern with NPAAs is the potential effect on crop yield, and whether the NPAAs are taken up into the crop to enter the food chain.

The leguminous plant alfalfa (*Medicago sativa*) is the most important and widely grown forage crop in the world and, due to its high protein content, it is well suited for hay production and livestock pasture (Barnes, 1988; Bouton, 2012; Irwin et al., 2001). The irrigation of alfalfa with water containing cyanobacteria is a possible mechanism of exposure leading to potential toxicity to the plant and/or bioaccumulation of BMAA and DAB. Given the unknown toxicity of BMAA and DAB to alfalfa and their potential for bioaccumulation and health effects in humans, we aimed to investigate their effects on plant growth. Secondly, we aimed to examine the potential for BMAA and DAB to bioaccumulate in alfalfa.

2. Materials and methods

2.1. Reagents

L-BMAA, analytical solvents and other reagents were purchased from Sigma Chemical Co., MO, USA unless otherwise specified. BioSnacky Certified Organic Alfalfa *Medicago sativa (M. sativa)* seeds (A. Vogel, TG, Switzerland) were purchased locally. MLA algal growth medium (Bolch and Blackburn, 1996) was prepared using the method described by the Australian National Algae Culture Collection (CSIRO, 2006). Mass spectrometry reagents used in this study are outlined by Main and colleagues, including the same column (Main et al., 2018). The isomer L-2,4-DAB was purchased from Toronto Research Chemicals ON, Canada and the deuterated internal standard for L-DAB; −2,4-Diaminobutyric-2,3,3,4,4-d5 (d5–2,4-DAB) was purchased from CDN isotopes, QC, Canada. The standardised EZ:faast high-performance liquid chromatography (HPLC) free amino acid derivatisation kit was purchased from Phenomenex Inc. CA, USA.

2.2. Experimental methods

2.2.1. Toxicity testing

The alfalfa seeds were germinated overnight on Whatman no. 1 90-mm filter paper (Whatman, Middlesex, U.K) in a Petri dish (10 cm diameter) with 5 mL MLA growth medium. Following germination, seeds of uniform size were selected for NPAA treatment.

To test the toxicity of the NPAAs on *M. sativa* seeds, each NPAA was diluted using MLA medium. For each NPAA the following concentrations were used: 300 μM, 500 μM, 1000 μM, 1500 μM and 2000 μM. MLA medium was used as a control. The pH of each solution was measured and adjusted with 0.01 M NaOH and 0.01 M HCl to match to the pH 7 control medium. Whatman GF/C filter papers (47-mm diameter) were placed in 50-mm Petri dishes, and dosed with 2 mL of the appropriate treatment. Five germinating *M. sativa* seeds were then placed on each filter paper. All samples were placed, in random order, under cool white fluorescent lighting with a 14:10-hour light: dark photoperiod at 25 °C for four days. On day three of treatment, 0.5 mL of Milli-Q (Millipore, MA, USA) water was added to each sample to compensate for evaporation and keep the filter paper moist. At least six replicates were measured, amounting to a total sample size of ≥30, for each experimental condition.

2.2.2. Protein hydrolysis and amino acid derivatisation

To investigate bioaccumulation, nine dishes of alfalfa samples (45 seedlings total) for each treatment (MLA control, 1800 μM BMAA, 1470 μM DAB, based on preliminary estimates of the respective IC50s) were prepared for hydrolysis using the method outlined in 2.2.1. Following the 4-day treatment, alfalfa roots and shoots were washed with ultrapure water (Sartorius arium® pro VF-B system) to ensure the removal of treatment solution from tissues. Roots and shoots were washed once during removal from treatment dishes for 10 min and three times totally submerged in 5 mL for 5 min each. Once washed, samples were snap frozen in liquid nitrogen, lyophilised and dry weights recorded. Tissue homogenisation was performed utilising cryo-bead-milling within a Retsch MM400 homogeniser at 30 Hz for three, one-minute cycles with cryo-freezing between intervals. The canisters were warmed externally with warm water, then 500 μL of water with 0.05% Triton X-100 was added to each canister. Manual solubilisation was performed and samples were transferred to a fresh tube. Between samples, canisters were first cleaned with Pyroneg (Diversey, SC, USA) to solubilise any remaining detritus. Then, canisters were washed with tap water, followed by rounds of clean methanol submersion and subsequent ultrapure water washing. Samples were then sonicated (Vibra-Cell, Sonics & Materials, CT, USA) on ice at 80% intensity for three, thirty-second intervals with insoluble material pelleted at 20,000 g for 20 min. The supernatant was transferred to new tubes and the protein was

precipitated with five volumes of 10% Trichloroacetic acid (TCA)-acetone (10:90 TCA to acetone ratio) for one hour at 4 °C then centrifuged at 20,000 g for 20 min. Precipitation was repeated twice, then the supernatants were combined and vacuum dried. The insoluble protein fraction was subjected to hydrolysis with 6 M HCl under nitrogen atmosphere at 110 °C for 16 h; the hydrolysed amino acids were then solubilised in 20 mM HCl. Both fractions were passed through a 0.2 μm centrifuge filter (Corning® Costar® Spin-X®, *#CLS8169,* purchased through Sigma-Aldrich).

Amino acid derivatisation was performed on sample fractions with a solid phase extraction clean up through the EZ:faast kit. This method uses propyl chloroformate to derivatise as well as a combination of proprietary solid phase extraction and solvent-based cleaning to reduce matrix-associated suppression and interference. We modified the standard procedure by incorporating our own internal standard (ITSD) of deuterated L-2,4-DAB spiked into every sample at 1 ppm. Standard curves were constructed using BMAA that was subsequently combined with internal standard and derivatised. For a comprehensive description of the methodology see Esterhuizen-Londt et al. (2011a).

2.2.3. Mass spectrometry

Sample analysis was performed using the assay developed by Main et al. (2018). Samples were injected on a 6490 triple-quadrupole mass spectrometer with a 1290 Infinity ultra-high performance liquid chromatography (UHPLC) (Agilent, CA, USA) (Table 1). The chromatography column was a Phenomenex Kinetex 150 × 2.1 mm 1.7 μm C18 UHPLC, at 37 °C with a flow rate of 250 μL min^{-1}. The mass-spectrometer (MS) was operated in positive data acquisition mode under ESI (capillary/nozzle: 3000/1500v), drying and sheath gas of 250 °C at 14/11 L min^{-1} and nebuliser pressure of 20 psi.

Five μL of sample was injected in technical triplicate per biological replicate and quantitation was performed in Agilent's Mass Hunter software version 7. Integration parameters were set to "MS-MS" with the use of the most abundant ion transition for quantitation; BMAA (333.01 > 187.1), DAB (333.01 > 273.1). The results were normalised against the ITSD to account for any matrix effects and quantitated against a standard curve from 1ppb to 2 ppm with a linear $r^2 > 0.99$ per batch. A blank surrogate with ITSD was included in each experiment.

The identity of the NPAAs was confirmed against BMAA and DAB standards, which also included known isomer AEG and the internal standard D5-DAB (Fig. 1), using methodology we have published previously (Main et al., 2018; Violi et al., 2019b).

2.2.4. Statistical analysis

The non-parametric Jonckheere-Terpstra test for ordered alternatives was used to assess dose-response. Equal variances were assessed using Levene's test. Statistical analyses were carried out with IBM SPSS Statistics 26 (IBM Corp., Armonk, New York).

3. Results

3.1. Effects of NPAAs on root and shoot growth in M. sativa

The effects of BMAA and DAB on root growth were compared in *M. sativa* over a range of concentrations after 4 days' treatment (Fig. 2A). A Jonckheere-Terpstra test for ordered alternatives showed that there was a statistically significant trend of lower median normalised root length scores with increasing concentration of BMAA ($T_{JT} = 4169$, $z = -11.809$, $p < 0.0005$) and DAB ($T_{JT} = 3614.5$, $z = -9.971$, $p < 0.0005$). In shoots, although to a lesser extent, there was also a significant trend of lower median normalised shoot length scores with increasing concentration of BMAA ($T_{JT} = 9587.5$, $z = -2.359$, $p < 0.018$) and DAB ($T_{JT} = 7061$, $z = -2.562$, $p < 0.010$) (Fig. 2B).

Table 1
Chromatographic conditions: mobile phase A; 0.1% (v/v) formic acid in water. B; 0.1% (v/v) formic acid in methanol. Column was equilibrated for two minutes post run to return to operating pressures of ~700 bar.

Time (min)	A (%)	B (%)
0	45	55
10	32	68
10.10	0	100
15	0	100
15.10	45	55

3.2. BMAA and DAB (free and protein-associated forms) in roots and shoots of M. Sativa

BMAA and DAB were detected in both free and hydrolysed fractions following separation by reversed phase chromatography and detection by mass spectrometry (Fig. 3).

Both free and protein-associated BMAA were detected in the roots and shoots of BMAA-treated plants (Fig. 3A and 3B). BMAA was predominantly in the free form with a 30–50-fold higher concentration of free BMAA over protein-associated BMAA. The amount of BMAA in the roots was more than tenfold higher than that in the shoots for both the free and the protein-associated forms. Similarly, DAB was present in roots and shoots in both free and protein-associated forms, with around tenfold more DAB in the roots than the shoots (Fig. 3C and 3D). Levels of free DAB in both the roots and shoots were 10–100-fold higher than the protein-associated form. Both free and bound DAB were also detected in the roots and shoots of control samples.

4. Discussion

4.1. Phytotoxicity

This is the first study to explore the effects of the cyanotoxins BMAA and DAB on forage crop growth and we have provided evidence that these NPAAs significantly inhibit the growth of alfalfa roots, and to a lesser extent, shoots (Fig. 2). In the case of BMAA, much lower concentrations (~85 nM) have induced oxidative stress responses in aquatic plants (Contardo-Jara et al., 2013; Esterhuizen-Londt et al., 2011b). However, it is likely that the antioxidant enzymes measured in these studies are more sensitive to toxicity-related changes than root growth and this may be due to enzymatic changes preceding, and contributing to, growth inhibition (e.g. cell wall-bound peroxidases cause cell wall lignification and growth inhibition) (Guidotti et al., 2013; Passardi et al., 2005). The growth inhibition observed is also consistent with the toxic effects observed for other cyanotoxins such as the hepatotoxin MC (Mitrovic et al., 2005) and neurotoxin anatoxin-a (Mitrovic et al., 2004). MC significantly inhibits root growth of the aquatic plant *Lemna minor* at 10 μg mL^{-1} (Mitrovic et al., 2005), which is roughly 10 μM when converted for comparison. The higher concentrations of BMAA and DAB required may be due to differences in sensitivities of the plant species examined, or to differing mechanisms of action between the cyanotoxins. *Lemna* may also be more sensitive because, as a floating plant, a higher proportion of the surface area is in contact with the test solution, compared with alfalfa where most of the contact surface is on the roots, and the shoot is generally held above the solution. The less significant dose-dependent response observed in shoots may be attributed to this positioning. MC and anatoxin-a are both potent, acute toxins, and alfalfa root length was significantly inhibited by these following a 7-day exposure to concentrations as low 0.005 μg mL^{-1} (Pflugmacher et al., 2006). Comparing these studies suggests BMAA and DAB are among the least phytotoxic cyanotoxins and therefore, irrigation water with cyanobacteria containing either MC or anatoxin-a is likely to have more impact on crop plants than cyanobacteria containing BMAA and/or DAB. Interestingly, the differing toxicity values may relate to the slow onset of symptoms in mammals (Spencer et al., 1987). MC and anatoxin-a exert acute toxic effects (Bláha et al., 2009) whilst BMAA has been

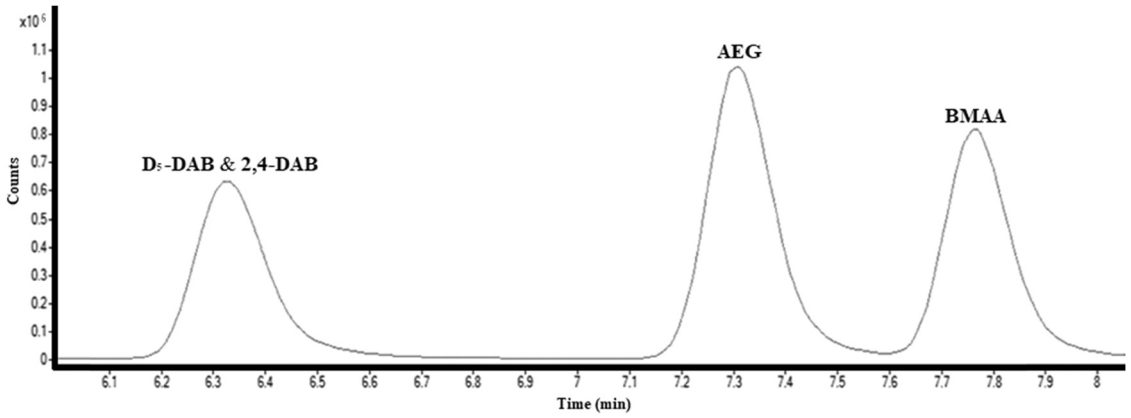

Fig. 1. Liquid chromatograph of a standard containing all isomers.

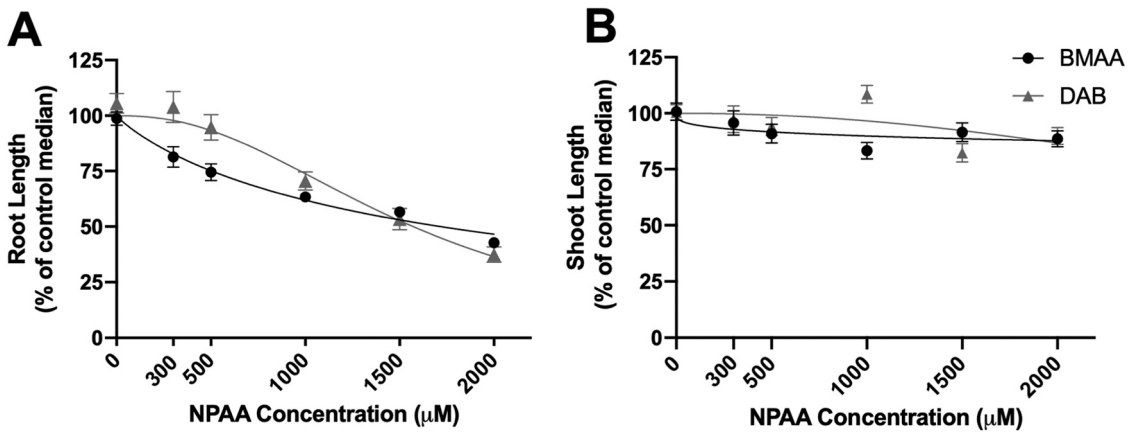

Fig. 2. Effects of BMAA and DAB on *M. sativa* root and shoot growth following 4-day treatment. (A) Root length. (B) Shoot length. Root and shoot lengths were normalised to the median length of the control group. Error bars show SEM. At least six replicates with five plants each were measured for each treatment concentration, with a sample size of ≥30 plants per concentration for each NPAA. The mean for each concentration is shown.

linked to the development of neurological disease in humans with a long-latency that has been attributed to protein misincorporation (Dunlop et al., 2013; Murch et al., 2004). We propose misincorporation of BMAA and DAB during protein synthesis as a potential mechanism of action against plants, a theory that will be discussed further below.

The observed results are comparable with the reported allelochemical activity of other NPAAs such as L-DOPA, an L-tyrosine analogue, and L-canavanine, an L-arginine analogue, in maize (*Zea mays*) and rice (*Oryza sativa*) seedlings respectively (Nakajima et al., 2001; Siqueira-Soares et al., 2013). L-DOPA inhibited maize root growth at 1000 μM (Siqueira-Soares et al., 2013), and rice leaf sheath growth was inhibited by L-canavanine at 500 μM (Nakajima et al., 2001). While these concentrations are similar to those observed in the current study, the phytotoxicity of BMAA and DAB is of much greater environmental concern due to the prevalence of cyanobacterial blooms.

The results of this study are of particular importance due to recent concerns that irrigation with cyanobacteria-contaminated waters may adversely affect crop yield (Pflugmacher et al., 2006). To date, BMAA and DAB have been detected across Africa, Europe, Australasia and the Americas in cyanobacteria, diatoms, dinoflagellates, aquatic plants and fish (Al-Sammak et al., 2014; Jiang et al., 2014; Jiao et al., 2014; Johnson et al., 2008; Lage et al., 2014; Violi et al., 2019b), and have been found in water samples at concentrations up to 25.3 μg L^{-1} BMAA and 14.7 μg L^{-1} DAB (Al-Sammak et al., 2014). However, experiments looking into the effects of cyanotoxins on crops have focused primarily on MC (Crush et al., 2008; Pflugmacher et al., 2007; Saqrane et al., 2009) and further investigations into BMAA and DAB phytotoxicity are warranted. A study on lettuce *(Lactuca sativa)* and arugula *(Eruca sativa)* observed an inhibitory response using both MC-containing and MC-free cyanobacterial extracts (Bittencourt-Oliveira et al., 2015) and BMAA and DAB could be responsible for the shortening of root lengths that was observed in MC-free extracts (Bittencourt-Oliveira et al., 2015). The evidence of phytotoxicity reported here contrasts with a recent investigation using wheat, where several morphological characteristics, including root length, were unaffected by seedling irrigation with BMAA containing water (Contardo-Jara et al., 2018). This difference can be attributed to the lower concentration used (10 μg mL^{-1} or ~85 μM) or the irrigation delivery system, which differs from that used in the present study. The prevalence of cyanobacteria that are able to produce BMAA and DAB suggests that NPAA toxicity to crops could be an important area for further research. Furthermore, the inhibition observed after treatment with both BMAA and DAB is consistent with the allelopathic roles they might play in plants that contain them. The presence of BMAA in all extant cycad genera strongly suggests that it evolved early and may have assisted in the evolutionary survival of the cycad, a plant dating back to the Mesozoic era (250-65 million years ago) (Brenner, 2003; Schneider et al., 2002).

4.2. Bioaccumulation and protein association

BMAA and DAB were detected in both the free and protein-bound, or associated, fractions of all NPAA-treated alfalfa (Fig. 3). The MS technique utilised for detection has previously been validated using cyanobacterial blooms and laboratory cultured cyanobacteria (Main et al.,

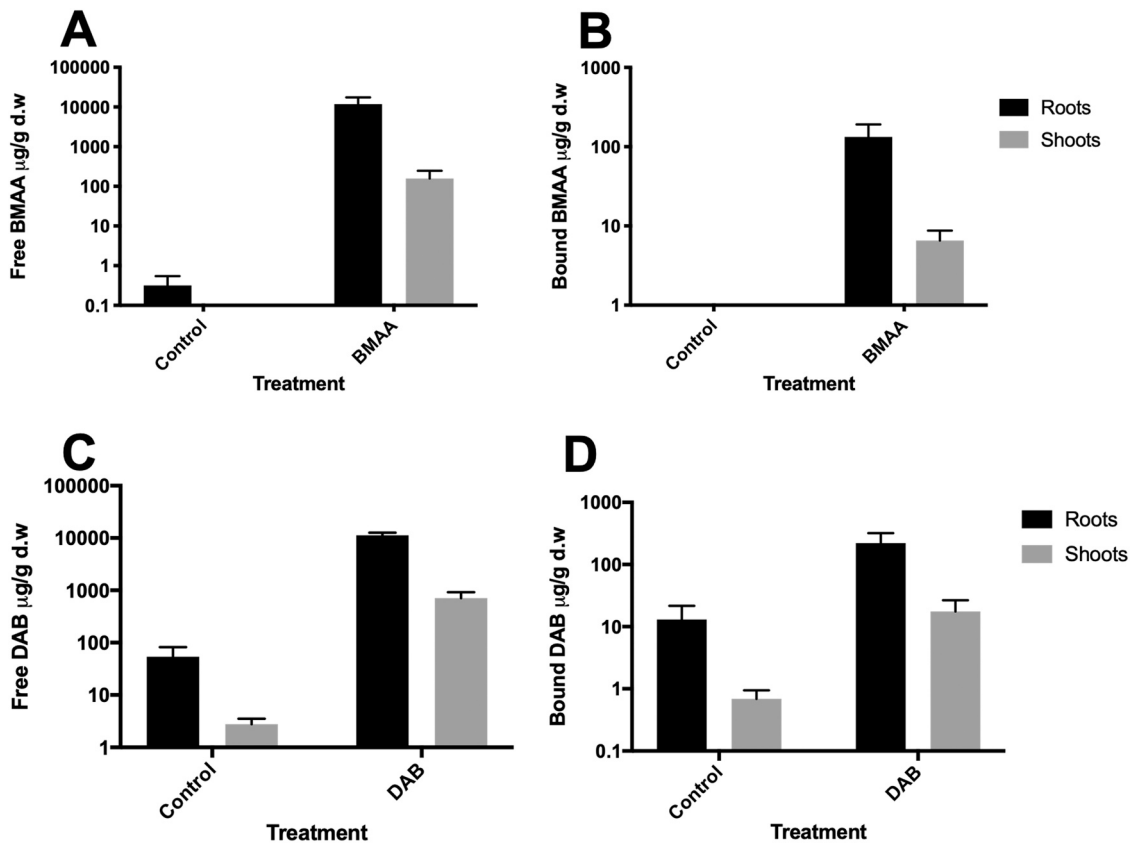

Fig. 3. Free and protein-bound BMAA (A–B) and DAB (C–D) in roots and shoots of *M. sativa* seedlings following 4-day exposure to 1800 µM BMAA and 1470 µM DAB. Concentrations are expressed as µg/g dry weight. Error bars show SEM. N = 3.

2018; Violi et al., 2019b). BMAA uptake has been reported in several terrestrial plant species (Contardo-Jara et al., 2018, 2014; Li et al., 2019) and this is the first study to demonstrate BMAA accumulation in the forage crop alfalfa. Furthermore, this is the first study to demonstrate DAB uptake in any plant species. This is significant due to the potential for these NPAAs to accumulate in food crops following exposure to irrigation water containing cyanobacterial blooms, thus entering the human food chain. In wheat, BMAA uptake was measured over 28 days at a treatment concentration of 100 µg mL^{-1}, which was considered an environmental "worst-case scenario" (Contardo-Jara et al., 2014). Bound BMAA concentration in the roots and shoots varied over the course of exposure with BMAA concentration higher in shoots on all days sampled except for day 14 where root BMAA content peaked. Free BMAA was below detectable limits during exposure (Contardo-Jara et al., 2014). This contrasts with the present study where free BMAA and DAB content was approximately 50-fold higher than that in the bound fraction. Free, but not bound, BMAA was also detected in root and shoot sections of spring onion (*Allium fistulosum*) and lettuce following prolonged exposure (60 days) with weekly BMAA irrigation (Esterhuizen-Londt and Pflugmacher, 2019). Li et al. detected both free and bound BMAA in Chinese cabbage and varying levels between different days of exposure suggest a potential explanation for the disparity in the literature (Li et al., 2019). It should also be noted that DAB was detected in both free and bound root fractions in the control samples. While some plant species do produce DAB (Nunn et al., 2010), there has been no evidence in the literature to suggest that alfalfa is a producer of DAB. However, alfalfa is a legume, and DAB has been detected in a variety of legume seeds (Bell, 1962a, 1962b; Nigam and Ressler, 1966; Pilbeam and Bell, 1979), and in the leaf, stem and root tissues of flatpea (*Lathyrus sylvestris*) (Shen et al., 1990). Furthermore, as cultures were not axenic the levels of DAB in the control may have originated from bacteria growing on the plant itself as the growth medium, MLA, can allow for bacterial growth (Bolch and Blackburn, 1996), although cultures were maintained using techniques to minimise bacterial contamination and growth.

Across all studies on BMAA and DAB uptake, there is contention about whether the contents of the bound fraction are truly incorporated into proteins or merely protein-associated (Dunlop et al., 2013; van Onselen et al., 2015, 2017). Contardo-Jara and colleagues reported protein-association of BMAA after only one day of exposure and argue that the absence of detectable free BMAA contradicts the presence of BMAA in the amino acid pool (Contardo-Jara et al., 2014, 2018). However, this theory is not supported by the current data, where free BMAA that may reside in the amino acid pool was detected. Despite this, the contents of the bound fraction cannot conclusively be attributed to misincorporation. Binding to receptor molecules has been proposed as an alternative mechanism for the protein-association observed in plants since BMAA has previously been shown to bind to NMDA receptors in neuronal cells (Contardo-Jara et al., 2014; Lobner et al., 2007). Overall, there is still need for further method validation for the determination of protein-bound and incorporated NPAAs. This is especially relevant for DAB, as this is the first study to detect bound DAB in plants. However, misincorporation is not the only proposed mechanism of NPAA phytotoxicity.

Oxidative stress has been suggested as another potential mechanism of toxicity, because BMAA exposure inhibits oxidative stress response enzymes in the aquatic macrophyte *Ceratophyllum demersum* (Esterhuizen-Londt et al., 2011b). Another study examined the oxidative stress responses of a range of aquatic plants and found that while response enzymes were inhibited in some plants, they were upregulated in others, even in the presence of BMAA and MC in combination (Contardo-Jara et al., 2013; Esterhuizen-Londt et al., 2011b). This suggests that plants such as *Lomariopsis lineata, Fontalis antipyretica*, *Riccia fluitans* and *Taxiphyllum barbieri* may be more tolerant of NPAA toxicity and could be

useful in remediating environments plagued by NPAA cyanotoxins (Contardo-Jara et al., 2013). In fact, a follow up study also conducted by the Pflugmacher lab used this method of analysis to confirm the suitability of the green alga *Aegagropila linnaei* for use in the Green Liver Systems® water purification system (Contardo-Jara et al., 2015; Pflugmacher et al., 2015).

5. Conclusions

We investigated the effects of BMAA and DAB on alfalfa growth and observed growth inhibition in the roots and, to a lesser extent, shoots. In addition, we studied the potential for these NPAAs to bioaccumulate in alfalfa and detected both compounds in the protein fraction. Together, these data highlight the significance of studying the effects of these two NPAAs in plant systems. Furthermore, their ability to accumulate in plants is a mechanism whereby these toxic NPAAs may enter the human food chain.

CReDiT authorship contribution statement

Kate Samardzic: Conceptualization, Investigation, Writing - original draft, Visualization, Writing – review & editing. **Joel R. Steele:** Investigation, Writing – review & editing. **Jake P. Violi:** Investigation, Writing – review & editing. **Anne Colville:** Methodology, Formal analysis, Visualization, Writing – review & editing. **Simon M. Mitrovic:** Conceptualization, Writing – review & editing. **Kenneth J. Rodgers**: Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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