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Vegetables cultivated with exposure to pure and naturally occurring β -N-methylamino-L-alanine (BMAA) via irrigation

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ABSTRACT

Bioaccumulation and biomagnification of β -N-methylamino-L-alanine (BMAA), a potent neurotoxin, has been demonstrated in various food webs. It is alarming as this intensification of BMAA will result in exposure to higher concentrations from a direct cyanobacterial source. As more food items are being identified as a source of BMAA and with the large variations in BMAA content, the aim of the present study was to evaluate BMAA uptake by, and accumulation in, two commonly consumed vegetables, *Lactuca sativa* and *Allium fistulosum*. Plants exposed to pure BMAA in controlled laboratory experiments, as well as vegetables naturally irrigated with water containing a BMAA producing cyanobacterial bloom were evaluated during growth and ripening. In the laboratory exposures, free BMAA was detected in both the edible ripe parts of *L. sativa* and *A. fistulosum* after 60 days of exposure to a total of 4.5 μ g BMAA. However, in the bloom exposure samples no BMAA could be detected in the ripe vegetables of *A. fistulosum*, *Cucurbita pepo*, or *Brassica rapa chinensis*. The study emphasises the need to further screen items for BMAA to understand the human exposure risk as well as the difference between BMAA uptake patterns with free BMAA and that contained in cyanobacterial cells.

1. Introduction

As a consequence of climate change and anthropogenic nutrient enrichment of surface waters, cyanobacterial blooms have increased both in incidence and intensity (reviewed in Scholz et al., 2017), releasing cyanotoxins with bloom senescence. Among the multitude of toxic substances produced by cyanobacteria (Lee et al., 2017), the non-proteogenic amino acid β -N-methylamino-L-alanine (BMAA) (Cox et al., 2005; Esterhuizen and Downing, 2008; Metcalf et al., 2008) is suspected to be a trigger for neurodegenerative illness (Murch et al., 2004; Holtcamp, 2012). Recently it was reported that BMAA is produced by marine diatoms and dinoflagellates (Jiang et al., 2014; Jiang and Ilag, 2014) as well contributing to a larger source of BMAA input into aquatic food chains. As cyanobacteria, dinoflagellates, and diatoms constitute a substantial portion of the primary producers in aquatic environments, assessing human exposure risk to BMAA becomes pertinent.

BMAA is water soluble and can be protein-associated leading to bioaccumulation and biomagnification from one trophic level to another within food webs. The first published account of BMAA bioaccumulation and biomagnification occurred on the island of Guam where the total BMAA was 8.7-fold higher in the roots of *Cycas*

micronesica, a cycad from which food items are made by the locals, than in the producing symbiont cyanobacteria *Nostoc* sp. (Cox et al., 2003; Murch et al., 2004). Biomagnification of BMAA was also shown in seafood from various regions (reviewed in Regueiro et al., 2017). These studies also illustrated that with consumption of organisms from higher trophic levels, the exposure to BMAA is substantially higher.

As BMAA has been detected in cyanobacteria, marine diatoms, and marine dinoflagellates, it is plausible that the human exposure risk to BMAA could be increased through surface water containing these microbes. Using surface water for irrigation of crops and bloom material as bio-fertilizer are existing practices in many countries e.g. China (Chen et al., 2006). At Lake Chao (China), which is characterized by persistent cyanobacterial blooms, lake water is commonly used for irrigation of agricultural commodities thereby possibly facilitating the transfer of cyanotoxins into the crops. For example, Pak choi (*Brassica rapa chinensis*), courgette (*Cucurbita pepo*), and spring onions (*Allium fistulosum* L.) cultivated by spray irrigation with water from Lake Chao exhibited intracellular microcystin concentrations above the acceptable daily intake advised by the World Health Organisation (Peuthert et al., 2015).

In laboratory experiments, BMAA was proven to accumulate in *Nasturtium officinale* (watercress) and *Daucus carota* (carrots)

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(Niyonzima, 2010), as well as in *Triticum aestivum* (wheat) (Contardo-Jara et al., 2014). In both these short-term studies, free and protein-associated BMAA could be detected in the plant material after exposure. However, in the long-term exposure experiment performed by Contardo-Jara et al. (2018), only protein-associated BMAA could be detected in *T. aestivum* wheat berries. This indicated that the form of BMAA, i.e. free or protein-associated, within plants changes with time and possibly the developmental stage of the plant.

Along with seafood, BMAA has also been detected in other dietary items such as eggs (Andersson et al., 2018), natural health products containing *Spirulina* from Canada (Glover et al., 2015), and some crop plants exposed under laboratory conditions (Niyonzima, 2010; Contardo-Jara et al., 2014, 2018). The human exposure risk via consumption may be underestimated and needs to be further explored. Due to various dietary preferences, it is furthermore necessary to consider the BMAA exposure risk via food items other than seafood and to understand whether a higher or lower BMAA exposure risk exists by following a specific diet, i.e. high seafood, vegetarian, vegan, etc. The present study intended to fill some of the knowledge gaps regarding BMAA uptake in long term laboratory exposures of commonly consumed ripe vegetables, to understand if the BMAA uptake pattern is different in diverse vegetables, and also examining how plausible BMAA uptake is in nature from a cyanobacterial bloom source containing a matrix of compounds including BMAA. The aims of the present study were therefore to (1) investigate the uptake and protein-association of BMAA in onion and lettuce exposed to pure BMAA in the laboratory from the germination of the seeds until ripe vegetables were produced and (2) cyanobacteria bloom water exposed ripe vegetables, i.e. irrigated with bloom water of which the bloom material tested positive for BMAA.

2. Methods and materials

2.1. Chemicals, reagents, and seeds

Allium (onion) and *Lactuca* (lettuce) spp. are among the most commonly cultivated and consumed vegetables worldwide, needing short cultivation periods of 55–60 days before harvesting and were chosen as test specimens. Lettuce (*Lactuca sativa*) and spring onion (*Allium fistulosum*) seeds were purchased from Sperli GmbH (Everswinkel, Germany). All chemicals were purchased from Sigma Aldrich unless specified otherwise.

2.2. Long-term laboratory exposure to pure BMAA

The seeds were surface sterilized with 3% H₂O₂ followed by a sterile distilled water wash. The seeds were imbibed overnight in artificial water (0.2 g/L CaCl₂, 0.103 g/L NaHCO₃, and 0.1 g/L sea salt), and were sprouted on tissue paper, which were soaked with artificial water, in petri dishes incubated at 21.5 ± 1 °C in the dark. The germinated seeds were planted in 150 mL sterile soil in Jiffy Pots (Jiffy Products International BV, Zuid-West, Nederland) in quintuplicate. Five seedlings per pot were planted per species respectively in 10 plant pots (5 control pots and 5 pots with BMAA exposure). They were irrigated with 10 mL of artificial media containing 50 µg BMAA/L once a week and 10 mL sterile artificial water without BMAA every other day (to avoid the soil from drying out). The pots were incubated at 21.5 ± 1 °C and a light intensity of 4400 lx (14 h:10 h light/dark cycle) for 60 days; i.e. 9 irrigations with BMAA, which resulted in exposure to 4.5 µg BMAA in total. The exposure concentration was selected based on calculated BMAA exposure in nature and to be comparable to previous studies (Niyonzima, 2010; Contardo-Jara et al., 2014, 2018). After 60 days of exposure, the plants were visually inspected for morphological differences between the exposed and unexposed groups. Thereafter, the plants were removed from the pots carefully, and washed in methanol and distilled water to remove remaining soil and surface attached

BMAA. The plants were sectioned into root and the shoot sections, weighed, snap frozen in liquid nitrogen, and were stored at −80 °C until BMAA extraction and analysis followed.

2.3. Long term natural exposure to BMAA

Lake Chao in the Anhui province of the people's republic of China was sampled in 2008 during a heavy bloom of *Microcystis aeruginosa* (Peuthert et al., 2015). The bloom material was collected and filtered through cellulose filters and dried at 70 °C for 12 h. *C. pepo* (courgette), *A. fistulosum* (spring onion), and *B. rapa chinensis* (Pak choi), grown on microagricultural fields on the shore of Lake Chao and irrigated with bloom containing water, were sampled at the same time. Five different samples from three agricultural fields were taken for each plant species. The samples were washed before being dried at 70 °C for 15 h and the dry weight was recorded. The samples were ground to a fine powder in liquid nitrogen and extracted as per Section 2.4.

2.4. BMAA extraction and quantification

Samples were extracted as detailed in Esterhuizen-Londt et al. (2011). Briefly, after drying and trituration, samples were dissolved 0.1 M trichloroacetic acid (TCA) and freeze-thawed (−40/23 °C) three times before ultrasonic treatment (Palsson ultrasonic cleaner) and homogenization (Intelli-Mixer) for 2 h. The samples were centrifuged at 20,800 × g for 10 min at 4 °C, before collecting the supernatant containing the free BMAA. The pellet was washed and centrifuged as stated before and the supernatant pooled with the first. After overnight liquid acid hydrolysis at 110 °C, the extract containing the protein-associated BMAA was pH adjusted to between 1 and 2 with NaOH before derivatization with a EZ: faast™ Amino acid analysis kit according to the manufacturers specifications (Esterhuizen-Londt et al., 2011). In order to quantify the BMAA, the samples were separated on a Phenomenex AAA-MS amino acid analysis column (250 × 2.0 mm) using an Agilent 1200 infinity series liquid chromatography system using 10 mM ammonium formate in water and 10 mM ammonium formate in methanol as mobile phases as detailed by Esterhuizen-Londt et al. (2011). For mass spectrometer detection, the BMAA parent compound with an *m/z* of 333 and product ions with *m/z* 273 and *m/z* 245 were monitored in multiple reaction monitoring mode. Drying gas temperature was set at a temperature of 340 °C and a flow of 8 L/min, sheath gas temperature was set to 380 °C and a flow of 11 L/min. The capillary voltage was set to 5000 V and the nozzle voltage was set to 1500 V. Nebulizer pressure was 35 psi. Limit of detection and quantification of the method was 0.5 pg and 2 pg on column, respectively.

2.5. Statistics

All statistical analyses were performed using SPSS software (IBM SPSS Statistics, Version 20). Normality and homogeneity of variance were tested using Shapiro-Wilk and Levene's test, respectively. Statistical significance between BMAA treatments and their corresponding controls were determined using the one-way analysis of variance (ANOVA) followed by Tukey's post-hoc or by the Mann-Whitney-U Test at a significance level of alpha = 0.05.

3. Results and discussion

3.1. Long term laboratory exposure to pure BMAA

After 60 days of BMAA exposure, the cultivars were examined and all plants appeared healthy exhibiting no signs of necrosis or wilting compared to the unexposed controls. Furthermore, no visible differences regarding the root morphology were observed after removal and cleaning of plants. Thus, from a morphological point of view, BMAA exposure did not influence the growth of the plants during the exposure

Fig. 1. Dry weights of the control and BMAA exposed groups of the A) *A. fistulosum* and B) *L. sativa* roots and shoots. Data represent mean dry weight (g) $\pm$ standard error.

period (Fig. 1). There were no significant differences between weights of the exposed roots and shoots and their corresponding controls of either the spring onions or the lettuce samples ($p > 0.05$).

No BMAA was detected in any of the control samples. Greater amounts of free BMAA was found in the spring onions compared to the lettuce, even though they were exposed to the same amount of BMAA (Fig. 2). In the shoots, free BMAA was only detected in *A. fistulosum* which was 6.6-fold less than the free BMAA detected in the roots of the spring onions. However, protein-associated BMAA was not detected in any of the samples after the long term exposure of 60 days.

Niyonzima (2010) was able to detect both free and bound BMAA in *D. carota* and *N. officinale* after 5 and 7 days of hydroponic exposure to 100 and 500 $\mu\text{g/L}$ BMAA. For both vegetables, Niyonzima (2010) found that the protein-associated fraction of BMAA was more than the free. By exposing *T. aestivum* to BMAA for 4 days, Contardo-Jara et al. (2014) could detect only protein-associated BMAA with exposure to 100 $\mu\text{g/L}$ BMAA, however, both free and bound BMAA could be detected with exposure to 1000 $\mu\text{g/L}$, in which case the free BMAA was 2.88-fold more than the protein-associated. In the current study, lower total BMAA concentrations were detected compared to those reported by Niyonzima (2010) even though the exposure period was much longer, i.e. 60 days. However, in contrast to the present study, Niyonzima's exposure was hydroponic in nature, excluding the possibility of soil binding which could have occurred in the present study and should be investigated in future. Also, in the case of Niyonzima's research, BMAA was not quantified in ripe crops, but in actively growing seedlings, which are assumed to take up and assimilate amino acids more actively due to requirements for growth. Contardo-Jara et al. (2018) conducted a long term BMAA exposure study with *T. aestivum*. After 205 days i.e. with BMAA exposure from seed germination to seed production, only

Fig. 2. Mean values of free and bound BMAA in the root and shoot sections A) *A. fistulosum* and B) *L. sativa* after 60 days of exposure (weekly irrigation with 50 $\mu\text{g/L}$ BMAA). Error bars represent standard error.

protein-associated BMAA could be detected in roots, shoots, and grains of *T. aestivum*. The protein-associated BMAA concentration was ten times higher in the wheat berries than in the roots and shoots. The group discussed that this may be because amino acids are transiently stored in seeds, which are then made available during germination. As the results of Contardo-Jara et al. (2014, 2018) were reported as a function of fresh weight, it is difficult to compare the total amounts taken up in *T. aestivum* versus those detected during the present study in *A. fistulosum* and *L. sativa*. However, it is clear that the distribution of free versus protein-associated BMAA is different not only from plant to plant but also during different growth stages.

Fruit and vegetable ripening represents the turning point between growth and development and the start of decomposition. During fruit or vegetable ripening, it has been reported that free amino acids increase significantly (Sorrequieta et al., 2010). In onions, free amino acids are translocated from dying foliage to the developing bulb (Nilsson, 1980) due to protein lysis and enzyme breakdown as a result of decreased metabolic function. This could also offer an explanation as to why only free BMAA was detected in *A. fistulosum* and *L. sativa* at ripening stage versus the seedling exposure studies of Niyonzima (2010) and Contardo-Jara et al. (2014) where mostly protein-associated BMAA was detected i.e. during active growth and protein synthesis. However, for all experiments, it is unknown if and how much of the previously taken up BMAA had been metabolized or covalently modified as this has been previously demonstrated to occur in macrophytes (Downing et al., 2015).

3.2. BMAA in naturally exposed crop samples

Both free and protein-associated BMAA was detected in the bloom material collected from Lake Chao (Table 1), from which water was used for the irrigation of the vegetables tested for BMAA in the present study. Assuming a bloom density of 20 mg/L , together with the total amount of BMAA detected in the bloom material (41.0 $\mu\text{g/g}$), this

Table 1

The BMAA detected in the bloom material and vegetables irrigated with cyanobacteria containing bloom water ($n = 5$).

Species name	Vegetable	Free BMAA (µg/g)	Protein-associated BMAA (µg/g)
<i>Allium fistulosum</i>	Spring onion	ND ^a	ND ^a
<i>Cucurbita pepo</i>	Courgette	ND ^a	ND ^a
<i>Brassica rapa chinensis</i>	Pak choi	ND ^a	ND ^a
<i>Microcystis aeruginosa</i>	Bloom material	35.015	6.074

^a ND Not detectable.

calculates to 820 ng BMAA/L. The estimated water needed to grow one head of lettuce and one spring onion until maturity is 111.4 L and 4.0 L, respectively (Mekonnen and Hoekstra, 2011). Therefore, the crops theoretically would have been exposed to 91.0 µg and 3.2 µg of BMAA each until ripening. Even though these theoretical concentrations are similar to that used in the present study (4.5 µg over a period of 60 days), BMAA could not be detected in any of the vegetable samples (*A. fistulosum*, *C. pepo*, and *B. rapa chinensis*) cultivated next to lake Chao (Table 1) as either the BMAA amounts were below the limit of detection or not present (Table 1).

Esterhuizen-Londt and Downing (2011) used a solid phase extraction method with a high recovery yield to concentrate BMAA from raw water impounds with blooms. However, they were not able to detect BMAA in any of the three concentrated water impound samples. They suggested that it could have been taken up, however, it is unknown if other organisms or substances have different affinities for BMAA and therefore can only be speculated on whether or not it could have been bound immediately after release, possibly also in the case of the naturally exposed vegetables. After uptake, the BMAA could have been metabolized or covalently modified as demonstrated by Downing et al. (2015) in *Ceratophyllum demersum*. Conversely it is also possible that the BMAA released from the bloom material was degraded and was never made available to the vegetables for uptake.

The BMAA released from the cyanobacteria could also have become bound to soil particles as it was previously reported to have a high affinity for binding to transition metal ions such as Zn^{2+} , Cu^{2+} , and Ni^{2+} (Nunn et al., 1989) which are abundant in soil and sediment particles. BMAA has previously been shown to be taken up by bacteria (Van Onselen et al., 2015). Therefore, due to the rich microbial community in soils, uptake by microbes cannot be excluded therefore reducing the BMAA made available for uptake into the vegetables. Also, in nature, toxin exposure never occurs with single pure compounds, but rather to a complex mixture and synergism of these compounds could have influenced the uptake of BMAA into these vegetables causing differences with the laboratory exposed samples.

Based on the current study it is not possible to understand why BMAA was detected in the vegetable exposed to pure BMAA in single and not in the bloom exposed samples. Albeit this was outside of the scope of the research, the clear difference between the controlled laboratory setting and open multifactor natural system is evident and this highlights the need for further investigation.

It is also important to note that nowadays, there is a more health conscious view on the traditional human diet with many people partially or completely changing to vegetarianism or even veganism, thereby consuming more vegetables and less meat and animal products. If no detectable levels of BMAA is present in naturally grown vegetables but substantial amounts in seafood, does this mean following a vegetarian diet would result in a lower exposure risk? Currently, there is no available information regarding BMAA in animals other than seafood. However, it has been reported that livestock can be exposed via biofilms on plants (McGorum et al., 2015) and that cyanobacteria are used in the preparation of some animal feeds (Yaakob et al., 2014). This, together with the irrigation of fields with cyanobacteria contaminated water and using cyanobacterial as biofertilizer, BMAA exposure via

food sources other than seafood becomes conceivable. Little information is available on BMAA in animal products, except for a study by Andersson et al. (2018) showing that BMAA transfer into eggs is possible and studies by Niyonzima (2010), Contardo-Jara et al., (2014, 2018), as well as the present study showing a risk via vegetables and grains.

BMAA is also not destroyed through cooking (Banack et al., 2006) as shown in cooked shrimp from Sweden (Jiang et al., 2014), which means that even conscious preparation of contaminated food cannot offer reliable protection. This suggests the need to screen further food items for BMAA and to establish a tolerable daily intake value for BMAA in food. Regueiro et al. (2017) suggested a tentative acute reference dose based on the toxicity results of Al-Sammak et al. (2015) as 200 µg/kg body weight. However, as more information becomes available regarding food items that contain BMAA, this will have to be re-evaluated.

4. Conclusion

This study shows that BMAA is taken up in crop plants grown from seed to consumable product after exposure to the pure toxin alone, however, BMAA could not be detected in ripe vegetables exposed to BMAA via bloom water. The amounts of BMAA detected in food items to date vary substantially. Comparing this study to existing literature, it becomes evident that BMAA taken up and bioaccumulation in plants differ substantially at the various growth phases. As more foods are being evaluated, more items are being identified as health risk factors, demonstrating that seafood is not the only possible route of exposure. The need to understand the process of BMAA uptake and incorporation together with the need for further testing of foodstuffs for BMAA is required, indicating the need for incorporating BMAA testing into food safety evaluations.

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Declarations of interest

None.

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