Protein association of β-*N*-methylamino-L-alanine in *Triticum aestivum via* irrigation

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Bioaccumulation of several cyanotoxins has been observed in numerous food webs. More recently, the neurotoxic, non-proteinogenic amino acid β-*N*-methylamino-L-alanine (BMAA) was shown to biomagnify in marine food webs. It was thus necessary to assess whether a human exposure risk *via* a terrestrial food source could exist. As shown for other cyanotoxins, spray irrigation of crop plants with cyanobacterial bloom contaminated surface water pose the risk of toxin transfer into edible plants parts. Therefore, in the present study, a possible transfer of BMAA into the seeds of one of the world's most widely cultivated crop plant *Triticum aestivum* *via* spray irrigation was evaluated. Wheat seeds were irrigated with water containing 10 µg BMAA L^{-1} until they reached maturity and were seed bearing (205 days). Several morphological characteristics, such as germination rate, number of roots per seedling, length of primary root and cotyledon, and diameter of the stems were evaluated to assess the effects of chronic exposure. After 205 days, BMAA bioaccumulation was quantified in roots, shoots, and mature seeds of *T. aestivum*. Neither adverse morphology effects were observed nor were free intracellular BMAA detected in any of the exposed plants. However, in mature seeds, 217 ± 150 ng protein-associated BMAA g FW^{-1} were detected; significantly more than in roots and shoots. This result demonstrates the unexpected bioaccumulation of a hydrophilic compound and highlights the demand to specify in addition to limit values for drinking water, tolerable daily intake rates for the cyanobacterial neurotoxin BMAA.

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1. Introduction

β-*N*-methylamino-L-alanine (BMAA) was first described by Vega and Bell (1967), who isolated the non-proteinogenic amino acid from the seeds of *Cycas micronesica*, a cycad indigenous to the west pacific island of Guam. In 2003, Cox et al. (2003) discovered that BMAA is produced by the nitrogen-fixing cyanobacterial endosymbiont of the genus *Nostoc*, which occur in the morphologically specialized positively geotropic roots of *C. micronesica.* However, against expectation due to its hydrophilic character, BMAA has been reported to be biomagnified within the Guam ecosystem from its cyanobacterial source to the cycad's reproductive organs, and further to animals that forage on the cycad seeds such as *Pteropus mariannus mariannus* (Murch et al. 2004a). The native Chamorro people traditionally consumed flour-based foods made from seeds of these cycads, as well as *P. mariannus mariannus,* commonly known as flying foxes, putatively resulting in the high incidence of the neurodegenerative illness amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS/PDC) (Vega and Bell 1967; Murch et al. 2004a; Pablo et al. 2009; Holtcamp 2012). The mechanism of BMAA neurotoxicity and other health risks BMAA exposure poses to humans are reviewed in Regueiro et al. (2017).

BMAA, which can be described as a methylated alanine, was shown to not only occur as a free amino acid in organisms but also in protein-associated form. Misincorporation into de novo formed proteins, seemingly in place of alanine or serine, has recently been evidenced and identified as a reason for the mal-functioning of the according proteins (Dunlop et al. 2013; Main et al. 2016). Furthermore, the protein association is said to facilitate the transfer of BMAA between the different trophic levels as described e.g. in the Guam food web, creating a toxin reservoir which slowly releases the toxin due to cerebral protein metabolism, thereby unfolding its negative effects time-delayed (Murch et al. 2004b).

Interestingly, it was later established that not only do endosymbiotic *Nostoc* spp. produce BMAA, but also diverse free-living cyanobacteria from different regions of the world (Cox et

al. 2005; Esterhuizen and Downing 2008; Metcalf et al. 2008). This, together with the fact that cyanobacteria occur ubiquitously, demonstrates not only a health threat for the aquatic ecosystem but also for humans as they can be directly exposed *via* drinking water or aerosols or indirectly *via* edibles in which BMAA might have been bioaccumulated (Banack et al. 2014; Contardo-Jara et al. 2014a; Jiang et al. 2014; Jonasson et al. 2010; Metcalf et al. 2008; Mondo et al. 2012; Niyonzima 2010).

Especially in the limnic system, cyanobacterial occurrence is troublesome as increased nutrient input from agricultural run-off and treated sewage promotes the formation of massive cyanobacterial blooms. The exceptionally high water temperatures seen during summer months in temperate regions, promoted by global warming, additionally boost the development of cyanobacterial blooms (reviewed in Scholz et al. 2017). Since cyanobacteria produce secondary metabolites exhibiting poisonous properties, the resulting hazard presents itself particularly when cells lyse, releasing these toxins. Besides the mentioned neurotoxin BMAA, cyanobacteria produce potent hepatotoxins, cytotoxins, and endotoxic lipopolysaccharides (Saqrane et al. 2009). As a consequence, special awareness and monitoring of harmful cyanobacterial blooms have existed for decades as indicated by established toxin limit values for drinking water by the world health organization (WHO), occasional swimming prohibition, as well as control and management strategies. These counteracting activities include e.g. physical and chemical bloom control or the development of green techniques (Green Liver Systems®) to remove toxins from water intended for spray irrigation use or drinking water production (Edwards and Lawton 2009; EPA 2014; Lawton and Robertson 1999; Pflugmacher 2015; Pflugmacher et al. 2015; WHO 1998). However, spray irrigation of cereal crops with raw untreated surface water remains a reality in many third world countries and therefore pose a possible transfer route for contaminants from the aquatic environment to the terrestrial ecosystem. Subsequently, the risk of biomagnification of these contaminants within food webs exist and thus presenting negative implications for human and livestock health. In arid, semi-

arid, and even temperate regions, field irrigation using surface water is the only alternative for lacking precipitation.

Bread wheat (*Triticum aestivum* L.) is the third most important cereal crop after maize and rice, with a global production of 650 million metric tons in 2010 (FAO 2013; IGC 2012), used for human consumption as well as for livestock feed. *T. aestivum* thrives and prospers preferentially in nutrient rich soils of temperate climate regions, therefore, the most important crop growing countries include the United States, Canada, Argentina, Australia, European Union, and China. Only special breeding enables the cultivation in e.g. Mexico, India, Pakistan, and parts of Africa (Cornell and Hoveling 1998; Lieberei and Residorff 2012; Popper et al. 2006). With more than 20% of the globally consumed calories, *T. aestivum* provides a considerable contribution to the nutrition of the worldwide human population (Bushuk and Rasper 1994; Brenchley et al. 2012). Concerning the global area used for agriculture, *T. aestivum* is the most frequently planted crop (FAO 2013) and depending on local climate and soil properties, crops have to be fertilized and irrigated. According to Mekkonen and Hoekstra (2011) bread wheat has a water footprint of around 1087 billion m^3 per year (Mekonnen and Hoekstra 2011). For 500 g of bread wheat, 650 virtual litres of water are necessary, of which around 19% represents spray irrigation with ground or surface water (Hoekstra et al. 2009; Mekonnen and Hoekstra 2011). The proportion of irrigation with ground and surface water highly varies between the different agricultural areas (Mekonnen and Hoekstra 2010). Concerning quality, security, and reliability of *T. aestivum* seeds as a comestible commodity, a disadvantage of field irrigation with surface water might be the above mentioned transfer and bioaccumulation of contaminants, in particular cyanotoxins. Numerous studies have shown that agricultural plants can take up, transport, and bioaccumulate cyanotoxins (Contardo-Jara et al. 2014a; Peuthert et al. 2007; Pflugmacher et al. 2007a, 2007b; Saqrane et al. 2009). For example, transfer of microcystins into *T. aestivum* seedlings and other edible plants such as e.g. *Medicago sativa, Brassica rapa chinensis, Allium fistulosum,* and *Cucurbita pepo* could be evidenced in

laboratory experiments as well as in the field after irrigation with contaminated lake water, respectively (Peuthert et al. 2007; Peuthert et al. 2015; Pflugmacher et al. 2007a). Additionally, high concentrations of heavy metals in mature *T. aestivum* seeds have previously been attributed to irrigation with contaminated water (Farid 2003; Jamali et al. 2009). These findings have brought about and highlighted the need to further investigate real life scenarios under defined experimental conditions. The aim of the present study was therefore to determine if BMAA would bioaccumulate in the seeds of *T. aestivum,* from seed germination to caryopsis maturation, *via* chronic irrigation with BMAA containing water at an environmentally relevant concentration.

2. Material and Methods

2.1 Exposure scenario

Two experiments were conducted; the first to investigate the effects of acute BMAA exposure on the germination and development of seeds into seedlings, and the second to investigate the bioaccumulation of BMAA in wheat with chronic exposure from seed germination to mature seed bearing plants.

T. aestivum seeds were surface sterilized with 3% H_2O_2 for 3 min before allowing them to imbibe water overnight at 20°C in the dark. Subsequently, 25 swollen seeds per replicate were placed in a petri dish and irrigated with 5 mL exposure media. The treatment group was irrigated with 10 µg BMAA L^{-1} and the control group with water, each in triplicate. In order to determine early morphological changes due to BMAA exposure the following parameters were recorded after five days of acute exposure: germination rate, number of roots per seedling, length of the primary root and length of the cotyledon.

In order to determine the transfer of BMAA into *T. aestivum* during long term exposure, polypropylene pots (20 × 20 × 27.5 cm), in quintuplicate, were filled with 3200 ± 5 g potting soil (Gardol, BAHAG AG), and twelve swollen wheat seeds were placed in each pot and

covered with 4 cm potting soil for both the control and the exposure groups. The control group was irrigated with tap water whereas the exposure group was irrigated with water containing 10 µg BMAA L^{-1}. The plants were cultivated at a constant temperature of 20 ± 1°C in a climate chamber at an initial photo period of 14:10 h and switched to 16:08 h light:dark cycle after 96 days to induce blooming. Plants were aerated manually from time to time by shaking a cardboard to facilitate pollination. The plant pots were irrigated according to requirements of the plants, i.e. once to twice a week for 26 weeks with an average of 273 mL per week. The wheat plants were harvested 205 days after the beginning of the exposure, i.e. after seeds were produced and matured. The exposure time of 205 days was selected in order to evaluate the uptake of BMAA after a full life cycle of the wheat plant i.e. until mature seeds were produced. The seeds were collected separately for every spike, snap-frozen in liquid nitrogen and stored at -80°C. Shoot samples included the whole aboveground part of the plant without the spike, whereas root samples consisted of the whole root system. Roots were washed in water to remove soil and surface adsorbed BMAA, respectively. Root and shoot samples were snap frozen in liquid nitrogen and stored at -80°C. In order to determine late morphological changes due to BMAA exposure, the following parameters were recorded at the end of the long term experiment: number of leafs per plant, length of the longest leaf, seed weight per spike and stem diameter for control and BMAA-treated plants.

2.2 BMAA extraction

BMAA extraction was carried out as described in Contardo-Jara et al. (2014a). Briefly, mature seeds, shoot, and root samples were ground to a fine powder using liquid nitrogen. For each sample, 0.2 g was mixed with 1 mL 0.1 M trichloroacetic acid (TCA), incubated for 30 min, and centrifuged at 4°C and 10,600 × g for 3 min. The supernatant was collected and the remaining pellets washed with 0.5 mL 0.1 M TCA, incubated for 30 min followed by centrifugation at 4°C and 20,800 × g for 3 min. The combined supernatants were analysed to determine the “free BMAA” tissue content. To determine the amount of protein-associated

BMAA, the above mentioned pellet was subsequently subjected to liquid acid hydrolysis in 1 mL of 6 M HCl containing 2% thioglycolic acid at 110°C for 24 h in an inert atmosphere. To remove solid residuals, the resulting extracts were filtered through 0.22 mm cellulose acetate filter. The extract pH was adjusted to between 1 and 2 with NaOH before derivatization. Extracts of free and protein-associated BMAA were derivatized using the EZ: faast™ amino acid analysis kit for LC–MS (Phenomenex). In short, the derivatization involved a concentration step using solid phase extraction, followed by sample clean-up and derivatization with a proprietary chloroformate derivative (Esterhuizen-Londt et al. 2011).

2.3 Quantitative Analysis

The derivatized amino acids were separated on a Phenomenex AAA-MS amino acid analysis column (2.0 × 250 mm, included in the kit) maintained at 35°C, using an Alliance® 2695 ultra-high pressure liquid chromatography system coupled with a Micromass Quattro micro™ (Waters) tandem mass spectrometer. An sample injection volume of 1 µL was used and the analytes were separated at a flow rate of 0.25 mL min^{-1} starting with 32% 10 mM ammonium formate in water (mobile phase A) versus 68% 10 mM ammonium formate in methanol (phase B). The gradient was linearly increased to 83% B within 13 min and then decreased immediately to 68% and maintained for 4 min. The derivatized BMAA (m/z of 333) was detected by selected ion recording (SIR) in positive mode. Using the above described gradient, BMAA showed a retention time of 8.2 min. Calibration was linear (R^2 = 0.999) based on BMAA standards in the range of 1 to 300 ng mL^{-1}. For both the free and protein associated BMAA fractions, the limit of detection was 1 ng mL^{-1} and limit of quantification 5 ng mL^{-1} derivatized BMAA, respectively. Quantification of derivatized BMAA was conducted with the internal standard Methionine-d3, allowing evaluation of derivatization efficiency. For verification purposes several samples were additionally measured in the multiple reaction monitoring (MRM) mode calculating the ratio of the product ions m/z 273 and m/z 245 (Esterhuizen-Londt et al. 2011).

2.4 Statistical analysis

Statistical analysis was conducted using Graph Pad Prism 6™ and significance was set at an alpha value of 0.05. The data were tested for homogeneity and normality using Levene's and Shapiro-Wilks tests. Statistical differences in germination rate, number of roots per seedling, length of the primary root, length of the cotyledon, number of leafs per plant, length of the longest leaf, and stem diameter between control plants and plants irrigated with 10 µg L^{-1} BMAA were tested by t-test using. BMAA content in the different plant tissues (shoot, root, and mature grain) irrigated with 10 µg L^{-1} BMAA were statistically compared using Kruskal Wallis test followed by Bonferroni post hoc test.

3. Results

3.1 Morphology

For the germination experiment, irrigation with 10 µg BMAA L^{-1} had no statistical effect on the germination rate and the number of roots per seedling. Compared to control plants, neither the length of the primary root nor the length of the cotyledon of the BMAA treated seeds displayed significant differences ($p > 0.05$).

For the chronically exposed plants, grown from germinating seeds to seed bearing maturity, no differences were found in the number of leafs per plant, the length of the longest leaf, the diameter of the stems, or the weight of the grains per spike compared to control plants after a 205 day exposure period ($p > 0.05$).

3.2 Uptake and incorporation of BMAA

No BMAA was detected in mature seeds, shoots, and roots of the control plants. Additionally, no free BMAA was detected in any of the exposed plant parts tested. However, protein-associated BMAA was detected in equal amounts in roots (25 ± 2 ng g FW^{-1}) and shoots (22 ± 19 ng g FW^{-1}) (Figure 1; $p > 0.05$). Seeds produced by the mature *T. aestivum* plants,

displayed a ten-fold higher concentrations compared to roots and shoots, with 217 ± 150 ng g FW^{-1} protein-associated BMAA (Figure 1; $p < 0.05$).

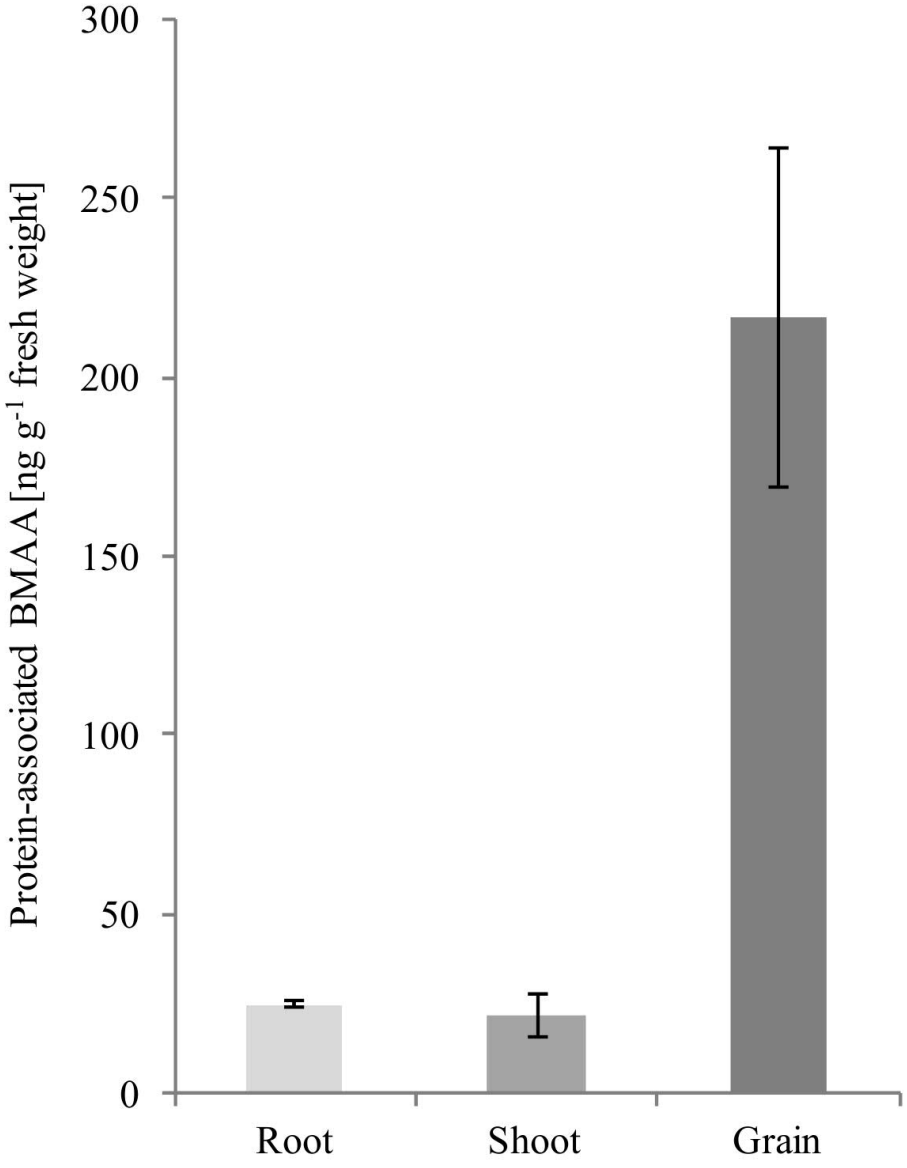

Figure 1. Protein-associated BMAA quantified in roots, shoots, and seeds of *T. aestivum* cultivated under chronic irrigated with 10 µg L^{-1} BMAA for 205 days. Data is presented as mean BMAA (ng) per plant fresh weight ± standard error (n=5).

4. Discussion

Since the majority of research studies have reported only intracellular BMAA concentrations of bloom material or cyanobacterial strains in culture (reviewed in Faassen

2014), concluding on extracellular BMAA in the water column and hence environmental relevance of the BMAA concentration (10 µg L^{-1}) used in the present study is challenging but crucial. Esterhuizen and Downing (2011) detected no extracellular BMAA in water supplies with incidence of cyanobacterial blooms, despite high concentrations in according bloom material (Esterhuizen and Downing 2008). Based on BMAA concentrations reported in laboratory cultures and environmental isolates (Cox et al. 2005; Metcalf et al. 2008) together with the typical bloom densities, Contardo-Jara et al. (2014b) concluded on 100 µg free BMAA L^{-1} as an environmental worst-case scenario. In the present study, to assess the possibility of bioaccumulation even from a mild bloom, a 10-fold lower concentration was selected.

4.1 Morphology

For the plants exposed to BMAA during germination and also for those chronically exposed, no morphological differences could be detected. Similarly, experiments with the edible plants *Nasturtium officinale* and *Daucus carota* cultivated in a hydroponic agar media containing 100, 300, and 500 µg L^{-1} BMAA over a time period of seven and nine days, respectively, also yielded no morphological changes regarding length of leaf, stem, root, or fresh weight (Niyonzima 2010). Morphological changes of crop plants during maturation or just before harvest will always be a first indication of possible damage of crop plants due to multiple reasons, such as droughts and floods, nutrient-poor conditions or other negative soil properties, pests, or contact with damaging agents, such as e.g. man-made contaminants or natural toxins (Martinelli et al. 2014; Mendelsohn 2007; Pflugmacher et al. 2007b). Hence, from a morphological point of view, plants exposed in the present experiment had no indication of any dysfunction.

4.2 Uptake and incorporation of BMAA

Free BMAA could not be detected in any of the exposed plant parts tested, however, protein-associated BMAA was detected in the roots, shoots, and seed of chronically exposed

plants. Interestingly the seeds displayed a ten-fold higher concentrations compared to roots and shoots. Physiological and genetic evidence exists supporting amino acid uptake and transport in plants (Persson and Näsholm 2001). A direct binding of BMAA to amino acid membrane transporters and consequent uptake into the xylem of the plant can be assumed based on the chemical structure of the compound. For the plant family Poaceae, a proportion of 25 to 55% nitrogen in an organic bound form (15 to 35% amides and 10 to 20% amino acids) of all nitrogen-containing substances in the xylem sap has been reported, whereas the rest, amounting to 45 to 75%, accounts for nitrate as salt (Mohr and Schopfer 1992). As an organic nitrogen source, amino acids are taken up from the soil by the roots (Näsholm et al. 2009), transported in xylem sap to leaf tissue driven by transpiration processes, and stored in the form of proteins, called transient storage (Mechthild and Doris 2010). Only a very small fraction of free amino acids might flow directly to seeds and apical meristem, which would explain why free BMAA was not detected. Typically seeds require a rapid import, translocation, and export of large amounts of transiently stored amino acid in a short time. During germination, transiently stored amino acids are remobilized from cotyledons and endosperm, and released by proteolysis, therefore possibly rendering free BMAA which could be transferred to the next generation of plants. Therefore, it is plausible that BMAA may have been misincorporated into proteins intended for transient storage in seeds explaining the high concentration in the seeds relative to the roots and shoots.

To date, there are no studies available concerning the experimental uptake of BMAA into edible plant parts (seeds, bulbs, roots, tubers, etc.) of crop plants as a consequence of irrigation with BMAA containing water. In comparison to the BMAA concentrations reported for cycads seeds (90 µg g^{-1}) and flour prepared from said seeds (40 - 179 µg g^{-1}) (Murch et al. 2004b), the BMAA concentrations quantified in the *T. aestivum* seeds after 205 days of exposure in the present study are much lower. In the case of cycad seeds from Guam, the BMAA producing cyanobacteria lives symbiotically within the cycad roots, whereas, in the present study, *T.*

290 *aestivum* was irrigated with water containing as little as 10 µg BMAA L^{-1}. In the present study,
291 an average amount of 87.5 ± 5 ng of total BMAA could be quantified per mature wheat plant.
292 However, a total of 7098 ng were added over the period of 26 weeks. This amounts to 0.2% of
293 the total amount of added BMAA which could be accounted for. The total amount of BMAA
294 in the soil was not evaluated in the present study, and it is plausible that a significant amount
295 of BMAA could have adhered to the soil or microbially metabolised. However, as plants often
296 utilize amino acids taken up from the soil as a source of nitrogen (Näsholm et al. 2009),
297 metabolism in the plant could also be considered as a possible route whereby BMAA could
298 have been lost.

299 Numerous studies exist which focuses on BMAA detection in various aquatic food webs,
300 starting with cyanobacterial bloom material to different tissue material of consumers of first,
301 second, and third order (brain, muscle, blood, etc.) and possible accumulation pathways along
302 the according food web (Brand et al. 2010; Jiao et al. 2014; Jonasson et al. 2010; Lage et al.
303 2015). Table 1 summarizes BMAA tissue concentrations of animals, which are directly
304 consumed by humans, together with extrapolated values of putative intake rates of
305 corresponding meals. The total extrapolated BMAA dosages per meal varies highly for identical
306 species from µg to mg values, depending on geographical site, putative occurring
307 cyanobacterial blooms simultaneous to the sampling of specimen, as well as probably different
308 analytical detection methods (Banack et al. 2014; Brand et al. 2010; Jiang et al. 2014; Jiao et
309 al. 2014; Jonasson et al. 2010; Mondo et al. 2012). Furthermore, it should be considered that
310 the different species/comestibles indicated belong to different trophic levels, exhibiting varying
311 nutrition modes as well as distinct lifespans, which explains the high differences in the BMAA
312 tissue concentration of e.g. shark fin compared to shrimps (Brand et al. 2010; Jiang et al. 2014;
313 Mondo et al. 2012). In turn, species displaying the same feeding mode as the filter-feeders blue
314 mussel or oyster revealed equal amounts of BMAA tissue concentration from same sampling
315 sites and events (Jiang et al. 2014; Jonasson et al. 2010).

316 Table 1 here.

317 Furthermore, it should be mentioned that the comestibles listed in table 1 are not
318 consumed on a daily bases and might vary considerably based on different global traditions
319 compared to products made out of *T. aestivum* seed flour. For European countries the
320 recommended daily intake rate of carbohydrates is estimated at around 1000 calories, which
321 corresponds to 250 g of bread wheat per day. Supposing that *T. aestivum* is the only
322 carbohydrate source and contaminated with BMAA (based on the results from the present
323 study), this would correspond to a total of 54 µg of BMAA consumed per day, or rather
324 exposure to 50 µg of BMAA assuming the oral bioavailability of 93% as reported by Duncan
325 et al. (1991). According to the WHO, a tolerable daily intake rate (TDI) is the amount of a
326 potentially harmful substance that can be consumed daily over a lifetime with negligible risk
327 of adverse health effect (WHO 1998). Assuming an average body weight of 60 kg per person,
328 the BMAA intake per day would corresponds to 0.9 µg kg^{-1} body weight. Al-Sammak et al.
329 (2015) reported that the lowest observed adverse effect level (LOAEL) for BMAA in mice is 2
330 mg g^{-1} body weight. However, since no limit or recommended value for TDI exist for BMAA,
331 it is difficult to conclude on the possible hazard this poses for human health. In contrast, for the
332 cyanobacterial hepatotoxin microcystin the WHO recommends a TDI of 0.04 µg kg^{-1} body
333 weight (Peuthert et al. 2015), hence 22-fold lower than the daily BMAA intake calculated from
334 the results of the present study. However, mode of toxic action as well as chemical
335 characteristics highly vary between those two cyanotoxins. Keeping in mind the possible
336 protein-association of BMAA, and the putative toxin reservoir which might be formed, as well
337 as missing biotransformation attempts, exposure to BMAA *via* edibles should not be
338 underestimated.

339 **5. Conclusion**

340 In conclusion, the presented study of *T. aestivum* chronically irrigated with BMAA over
341 its whole lifecycle, from germination to plant maturation and seed development, can be

considered as a plausible environmental scenario since spray irrigation with surface water is a common practice in agriculture and the applied BMAA concentration in the irrigation medium is of environmental relevance. BMAA did not affect the growth or other morphology of *T. aestivum*; however, significant amounts of the BMAA were accumulated in mature seeds, therefore, morphological appearance of crop plants cannot always be used as a gauge of exposure risk. The results presented demonstrate the risk of BMAA transfer from surface water into edible plant parts, highlighting furthermore the need to establish TDI rates for toxins of cyanobacterial origin not only in drinking water, but also in food.

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6. References

Al-Sammak MA, Rogers DG, Hoagland KD. 2015. Acute β-N-methylamino-L-alanine toxicity in a mouse model. J Toxicol. 2015:1-9.

Banack SA, Metcalf JS, Bradley WG, Cox PA. 2014. Detection of cyanobacterial neurotoxin β-*N*-methylamino-L-alanine within shellfish in the diet of an ALS patient in Florida. Toxicon 90:167–173.

Brand LE, Pablo J, Compton A, Hammerschlag N, Mash DC. 2010. Cyanobacterial blooms and the occurrence of the neurotoxin beta-N-methylamino-L-alanine (BMAA) in South Florida aquatic food webs. Harmful Algae 9:620–635.

366 Brenchley R, Spannagl M, Pfeifer M, Barker GLA, D'Amore R, Allen AM, McKenzie N,
367 Kramer M, Kerhornou A, Bolser D, Kay S, Waite D, Trick M, Bancroft I, Gu Y, Huo N,
368 Luo MC, Sehgal S, Gill B, Kianian S, Anderson O, Kersey P, Dvorak J, McCombie WR,
369 Hall A, Mayer KFX, Edwards KJ, Bevan MW, Hall N. 2012. Analysis of the bread wheat
370 genome using whole-genome shotgun sequencing. Nature 491:705–710.

371 Bushuk W, Rasper VF. 1994. Wheat – Production, properties and quality. Glasgow: Chapman
372 and Hall.

373 Contardo-Jara V, Otterstein SKB, Downing S, Downing TG, Pflugmacher S. 2014b. Response
374 of antioxidant and biotransformation systems of selected freshwater mussels (*Dreissena*
375 *polymorpha, Anadonta cygnea, Unio tumidus,* and *Corbicula javanicus*) to the
376 cyanobacterial neurotoxin β-N-methylamino-L-alanine. Toxicol. Environ. Chem. 96:451–
377 465.

378 Contardo-Jara V, Schwanemann T, Pflugmacher S. 2014a. Uptake of a cyanotoxin, β-N-
379 methylamino-L-alanine, by wheat (*Triticum aestivum*). Ecotox. Environ. Safe. 104:127–
380 131.

381 Cornell HJ, Hoveling AW. 1998. Wheat: Chemistry and utilization. Boca Raton: CRC Press.

382 Cox PA, Banack SA, Murch SJ. 2003. Biomagnification of cyanobacterial neurotoxins and
383 neurodegenerative disease among the Chamorro people of Guam. Proc. Natl. Acad. Sci.
384 USA. 100:13380–13383.

385 Cox PA, Banack SA, Murch SJ, Rasmussen U, Tien G, Bidigare RR, Metcalf JS, Morrison LF,
386 Codd GA, Bergman B. 2005. Diverse taxa of cyanobacteria produce β-*N*-methylamino-L-
387 alanine, a neurotoxic amino acid. Proc. Natl. Acad. Sci. USA. 102:5074–5078.

388 Duncan MW, Villacreses NE, Pearson PG, Wyatt L, Rapoport SI, Kopin IJ, Markey SP, Smith
389 QR. 1991. 2-amino-3-(methylamino)-propanoic acid (BMAA) pharmacokinetics and blood-
390 brain barrier permeability in the rat. J Pharmacol Exp Ther. 258:27-35.

391 Edwards C, Lawton LA. 2009. Bioremediation of cyanotoxins. Adv. Appl. Microbiol. 67:109–
392 129.

393 [EPA] United States Environmental Protection Agency. 2014. Cyanobacteria and cyanotoxins:
394 Information for drinking water systems. EPA-810F11001.

395 Esterhuizen-Londt M, Downing TG. 2011. Solid phase extraction of β-N-methylamino-L-
396 alanine (BMAA) from South African water supplies. Water SA 37:523-528.

397 Esterhuizen M, Downing TG. 2008. Beta-N-methylamino-L-alanine (BMAA) in novel South
398 African cyanobacterial isolates. Ecotox. Environ. Saf. 71:309–313.

399 Esterhuizen-Londt M, Downing S, Downing TG. 2011. Improved sensitivity using liquid
400 chromatography mass spectrometry (LC-MS) for detection of propyl chloroformate
401 derivatised β-*N*-methylamino-L-alanine (BMAA) in cyanobacteria. Water SA 37:133–138.

402 Faassen EJ. 2014. Presence of the neurotoxin BMAA in aquatic ecosystems: what do we really
403 know? Toxins 6:1109–1138.

404 [FAO] Food and Agriculture Organization of the United Nations. 2013. FAO Statistical
405 Yearbook 2013, World food and agriculture. FAO, Rome.

406 Farid S. 2003. Heavy metal ions concentration in wheat plant (*Triticum aestivum* L.) irrigated
407 with city effluent. Pakistan. J. Sci. Ind. R. 46:395–398.

408 Hoekstra AY, Chapagain AK, Aldaya MM, Mekonnen MM. 2009. Water footprint manual:
409 State of the art 2009. Water Footprint Network, Enschede.

410 Holtcamp W. 2012. The emerging science of BMAA: Do cyanobacteria contribute to
411 neurodegenerative disease? Environ Health Persp. 120:110–116.

412 [IGC] International Grains Council. 2012. Five-year global supply and demand projections to
413 2017/18. London: International Grains Council.

Jamali MK, Kazi TG, Arain MB, Afridi HI, Jalbani N, Kandhro GA, Shah AQ, Baig JA. 2009. Heavy metal accumulation in different varieties of wheat (*Triticum aestivum* L.) grown in soil amended with domestic sewage sludge. J. Hazard. Mater. 164:1386–1391.

Jiang L, Kiselova N, Rosen J, Ilag LL. 2014. Quantification of neurotoxin BMAA (β-N-methylamino-L-alanine) in seafood from Swedish markets. Sci Rep. 4:6931.

Jiao Y, Chen Q, Chen X, Wang X, Liao X, Jiang L, Wu J, Yang L. 2014. Occurrence and transfer of a cyanobacterial neurotoxin β-methylamino-L-alanine within the aquatic food webs of Gonghu Bay (Lake Taihu, China) to evaluate the potential human health risk. Sci Total Environ. 468-469:457–463.

Jonasson S, Eriksson J, Berntzon L, Spacil Z, Ilag LL, Ronnevi LO, Rasmussen U, Bergman B. 2010. Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure. Proc. Natl. Acad. Sci. USA. 107:9252–9257.

Lage S, Annadotter H, Rasmussen U, Rydberg S. 2015. Biotransfer of β-*N*-methylamino-L-alanine (BMAA) in a eutrophicated freshwater lake. Mar Drug. 13:1185–1201.

Lawton L, Robertson PKJ. 1999. Physico-chemical treatment methods for the removal of microcystins (cyanobacterial hepatotoxins) from potable waters. Chem. Soc. Rev. 28:217–224.

Lieberei R, Reisdorff C. 2012. Nutzpflanzenkunde, eighth ed. Stuttgart: Georg Thieme.

Main BJ, Dunlop RA, Rodgers KJ. 2016. The use of L-serine to prevent β-methylamino-L-alanine (BMAA)-induced proteotoxic stress in vitro. Toxicon 109:7-12. doi: 10.1016/j.toxicon.2015.11.003.

Martinelli F, Scalenghe R, Davino S, Panno S, Scuderi G, Ruisi P, Villa P, Stroppiana D, Boschetti M, Goulart LR, Davis CE, Dandekar AM. 2014. Advanced methods of plant disease detection. A review. Agron. Sustain. Dev. 35:1-25.

438 Mechthild T, Doris R. 2010. Uptake and partitioning of amino acids and peptides. Mol Plant.
439 3:997-1011.

440 Mekonnen MM, Hoekstra AY. 2010. A global and high-resolution assessment of the green,
441 blue and grey water footprint of wheat. Hydrol. Earth Syst. Sci. 14:1259–1276.

442 Mekonnen MM, Hoekstra AY. 2011. The green, blue and grey water footprint of crops and
443 derived crop products. Hydrol. Earth. Syst. Sc. 15:1577–1600.

444 Mendelsohn R. 2007. What causes crop failure? Clim. Chang. 81:61–70.

445 Metcalf JS, Banack SA, Lindsay J, Morrison LF, Cox PA, Codd GA. 2008. Co-occurrence of
446 beta-N-methylamino-L-alanine, a neurotoxic amino acid with other cyanobacterial toxins in
447 British waterbodies, 1990-2004. Environ Microbiol. 10:702–708.

448 Mohr H, Schopfer P. 1992. Pflanzenphysiologie, fourth ed. Berlin: Springer.

449 Mondo K, Hammerschlag N, Basile M, Pablo J, Banack SA, Mash DC. 2012. Cyanobacterial
450 neurotoxin β-*N*-methylamino-L-alanine (BMAA) in shark fins. Mar Drugs. 10:509–520.

451 Murch SJ, Cox PA, Banack SA. 2004b. A mechanism for slow release of biomagnified
452 cyanobacterial toxins and neurodegenerative disease in Guam. Proc. Natl. Acad. Sci. USA.
453 101:12228–31.

454 Murch SJ, Cox PA, Banack SA, Steele JC, Sacks OW. 2004a. Occurrence of beta-methylamino-
455 L-alanine (BMAA) in ALS/PDC patients from Guam. Acta Neurol Scand. 110:267–269.

456

457 Näsholm T, Kielland K, Ganeteg U. 2009. Uptake of organic nitrogen by plants. New Phytol.
458 182(1):31-48. doi: 10.1111/j.1469-8137.2008.02751.x

459 Niyonzima FN. 2010. Bioaccumulation and ecotoxicology of β-methylamino-L-alanine
460 (BMAA) in model crop plants [master´s thesis]. Port Elizabeth (RSA): Nelson Mandela
461 Metropolitan University.

462 Pablo J, Banack SA, Cox PA, Johnson TE, Papapetropoulos S, Bradley WG, Buck A,
463 Mash DC. 2009. Cyanobacterial neurotoxin BMAA in ALS and Alzheimer's disease. Acta
464 Neurol Scand. 120:216–225.

465 Persson J, Näsholm T. 2001. Amino acid uptake: a widespread ability among boreal forest
466 plants. Ecol. Lett. 4:434–438.

467 Peuthert A, Chakrabarti S, Pflugmacher S. 2007. Uptake of microcystins-LR and -LF
468 (cyanobacterial toxins) in seedlings of several important agricultural plant species and the
469 correlation with cellular damage (lipid peroxidation). Environ. Toxicol. 22:436–442.

470 Peuthert A, Kun L, Baik S, Pflugmacher S. 2015. Transfer of cyanobacterial toxins into edible
471 plants *via* irrigation with lake water - A chinese case study. Aperito J. Aquat. Mar. Ecos.
472 1:101.

473 Pflugmacher S. 2015. Green liver concept and Green liver systems - A sustainable way for
474 future water purification. Aperito J. Aquat. Mar. Ecos. 1:102.

475 Pflugmacher S, Aulhorn M, Grimm B. 2007b. Influence of a cyanobacterial crude extract
476 containing microcystin-LR on the physiology and antioxidative defence systems of different
477 spinach variants. New Phytol. 175:482–489.

478 Pflugmacher S, Hofmann J, Hübner B. 2007a. Effects on growth and physiological parameters
479 in wheat (*Triticum aestivum* L.) grown in soil and irrigated with cyanobacterial toxin
480 contaminated water. Environ. Toxicol. Chem. 26:2710–2716.

481 Pflugmacher S, Kuehn S, Lee SH, Choi JW, Baik S, Kwon KS, Contardo-Jara V. 2015. Green
482 Liver Systems® for Water Purification - Using the phytoremediation potential of aquatic
483 macrophytes for the removal of different cyanobacterial toxins from water. Am. J. Plant. Sci.
484 6:1607–1618.

485 Popper L, Schäfer W, Freund W. 2006. Future of flour – A compendium of flour improvement.
486 Erling, Clenze.

Regueiro J, Negreira N, Carreira-Casaisa A, Pérez-Lamelaa C, Simal-Gándaraa J. 2017. Dietary exposure and neurotoxicity of the environmental free and bound toxin β-N-methylamino-L-alanine. Food Research International 100:1–13.

Saqrane S, Ouahid Y, El Ghazali I, Oudra B, Bouarab L, del Campo FF. 2009. Physiological changes in *Triticum durum*, *Zea mays*, *Pisum sativum* and *Lens esculenta* cultivars, caused by irrigation with water contaminated with microcystins: a laboratory experimental approach. Toxicon 53:786–796.

Scholz SN, Esterhuizen-Londt M, Pflugmacher S. 2017. Rise of toxic cyanobacterial blooms in temperate freshwater lakes: causes, correlations and possible countermeasures. Toxicol. Environ. Chem. 99(4):543-577.

Vega A, Bell EA. 1967. α-Amino-β-methylaminopropionic acid, a new amino acid from seeds of *Cycas circinalis*. Phytochemistry 6:759–762.

[WHO] World Health Organization. 1998. Guidelines for drinking-water quality, second ed. Health criteria and other supporting information, Geneva.

Figure title:

Figure 1. Protein-associated BMAA quantified in roots, shoots, and seeds of *T. aestivum* cultivated under chronic irrigated with 10 µg L^{-1} BMAA for 205 days. Data is presented as mean BMAA (ng) per plant fresh weight ± standard error (n=5).

507 Table 1. Maximal detected BMAA concentration [µg g^{-1} WW*] in different comestibles, typical portion per
508 corresponding meal or daily intake rate [g], extrapolated BMAA dosage per meal or daily intake rate [µg or mg]
509 and corresponding reference. Only data from selected animal species from corresponding references are indicated.

Comestible	**BMAA in µg g^{-1} WW***	**Typical portion per meal**	**Extrapolated BMAA dosage per meal**	**Reference**
Shrimp	1156 µg/g	100 g	115.6 mg	Brand et al, 2010
Oyster	275 µg/g	100 g	27.5 mg	Brand et al, 2010
Blue mussel	(0.2 µg/g DW) *0.02 µg/g	100 g	2 µg	Jonasson et al. 2010
Oyster	(0.14 µg/g DW) *0.014 µg/g	175 g	1.4 µg	Jonasson et al. 2010
Shark fin[a]	1836 µg/g	10 g	18.4 mg	Mondo et al. 2012
Lobster	(19.3 µg/g DW) *1.9 µg/g	200 g	380 µg	Banack et al. 2014
Oyster	0.7 µg/g (raw)	100 g	70 µg	Jiang et al. 2014
Blue mussel	0.9 µg/g (raw)	175 g	158 µg	Jiang et al. 2014
Shrimp	0.5 µg/g (cooked)	100 g	50 µg	Jiang et al. 2014
Fish muscle	(35.9 µg/g DW) *3.5 µg/g	200 g	718 µg	Jiao et al. 2014
Bread wheat	0.22 µg/g	250 g (Daily intake rate)	55 µg	Present study

510 * assuming a WW/DW factor of 10

511 [a] from animal welfare point of view, shark fins should not be consumed in any form, as soup or dietary supplement