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Article

Azolla as a Safe Food: Suppression of Cyanotoxin-Related Genes and Cyanotoxin Production in Its Symbiont, *Nostoc azollae*

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Abstract: The floating freshwater fern *Azolla* is the only plant that retains an endocyanobiont, *Nostoc azollae* (aka *Anabaena azollae*) during its sexual and asexual reproduction. The increased interest in *Azolla* as a potential source of food, and its unique evolutionary history have raised questions about its cyanotoxin content and genome. Cyanotoxins are potent toxins synthesized by cyanobacteria which have an anti-herbivore effect, but also have been linked to neurodegenerative disorders including Alzheimer's and Parkinson's diseases, liver and kidney failure, muscle paralysis and other severe health issues. In this study, we investigated 48 accessions of the *Azolla-Nostoc* symbiosis for the presence of genes coding microcystin, nodularin, cylindrospermopsin and saxitoxin, and BLAST analysis for anatoxin-a. We also investigated the presence of the neurotoxin β -N-methylamino-L-alanine (BMAA) in *Azolla* and *N. azollae* through LC-MS/MS. The PCR amplification of saxitoxin, cylindrospermopsin, microcystin, and nodularin genes showed that *Azolla* and its cyanobiont *N. azollae* do not have the genes to synthesize these cyanotoxins. Additionally, the matching of the anatoxin-a gene to the sequenced *N. azollae* genome does not indicate the presence of the anatoxin-a gene. The LC-MS/MS analysis showed that BMAA and its isomers AEG and DAB are absent from *Azolla* and *Nostoc azollae*. *Azolla* therefore has the potential to safely feed millions of people due to its rapid growth while free-floating on shallow fresh water without the need for nitrogen fertilizers.

Keywords: *Azolla*; *Nostoc*; *Anabaena*; symbiosis; BMAA; microcystins; nodularin; anatoxin-a; cylindrospermopsin; saxitoxin

1. Introduction

Azolla Lam. is the only plant with a permanent nitrogen-fixing cyanobacterial symbiont (cyanobiont) that has chains of cells (filaments) comprising photosynthetic vegetative cells and thicker-walled heterocysts that contain the nitrogen-fixing enzyme nitrogenase [1]. The cyanobiont has been assigned to both *Anabaena azollae* and *Nostoc azollae* because its morphology resembles free-living species of both genera, including their change into motile hormogonia and akinetes (resting cells) that ensure survival during stressed conditions [2].

Genetic and paleontological data indicate that the *Azolla* – *N. azollae* symbiosis originated 80 million years ago in North America following Whole Genome Duplication (WGD) that increased the

genome of *Azolla*'s immediate ancestor [3]. *Nostoc azollae*'s subsequent coevolution with *Azolla* caused extensive changes in the cyanobiont's genome compared to free-living species of *Anabaena* and *Nostoc* [3–8]. Some changes involved the upregulation of genes that enhanced *N. azollae*'s sequestration of atmospheric nitrogen and provision of nitrogen-based compounds to *Azolla*, increasing the plant's speed of growth free-floating on fresh water. The downregulation, loss, or conversion to pseudogenes of other genes changed *N. azollae*'s ancestors from independent free-living organisms into obligate endosymbionts, reflecting *N. azollae*'s permanent location inside the leaves and female megasporocarps of *Azolla*. These included genes that previously expressed proteins involved in the synthesis of carotenoid and chlorophyll pigments, so that *A. azollae* is reliant on *Azolla*'s cellular pigments for protection against photooxidative damage [6].

Colonies of *N. azollae* live in specialized cavities in *Azolla*'s dorsal floating leaves, providing nitrogen-based nutrients to the plant that enable it to double its biomass in less than two days while free-floating on fresh water [9,10]. As a result, *Azolla* has been used for hundreds of years in India and the Far East as a nitrogen biofertilizer for paddy rice, reducing mosquito breeding populations by 95% [11–13] and emissions of the potent greenhouse gas methane from paddies by 25–50% [14–16]. *Azolla* also provides livestock feed, biofuel and biofertilizer for other plants, alleviating shortages of the 'three Fs' that increasingly threaten food supplies globally: feed, fuel, and fertilizer. It absorbs and removes phosphates and nitrates from water contaminated by chemical fertilizers, industrial pollutants, and animal and human waste that trigger toxic cyanobacterial (aka blue-green algal) blooms in rivers and lakes. The symbionts' combined CO₂ sequestration also increases *Azolla*'s carbon capture, so that it can sequester large amounts of atmospheric CO₂, with the plants being compressed and stored to reduce anthropogenic climate change through Carbon Capture and Storage (CCS). *Azolla* can, therefore, mitigate many of the threats arising from a Perfect Storm as our population increases by more than a million every three days. Its remarkable properties are increasingly recognised, and it has designated as a unique superorganism by [1].

Azolla has the potential to help feed millions of people because of its rapid growth, ease of outdoor cultivation in tropical and temperate regions, plus global production using the indoor *Azolla* Biosystem described by Bujak & Bujak (2020) in 'The *Azolla* Story' [2]. The use of *Azolla* for human consumption was thought to be limited by its high total polyphenolic content (TPC), but Winstead et al. (2024) [17] showed that the TPC of raw *Azolla caroliniana*, which is native in the eastern United States, have only 4.26 g gallic acid equivalent (GAE) kg⁻¹ DW, and that simple cooking methods can decrease TPC in all *Azolla* species. They also demonstrated that its protein content is 19% DW and its apparent protein digestibility is 78.45%, with a yield 173 g FW m⁻² day⁻¹ and 5.53 g DW m⁻² day⁻¹, confirming *Azolla*'s potential for cultivation and domestication as a nutritious food. This raises the question of whether *Azolla* is safe to eat because of the presence of harmful cyanotoxins in many cyanobacteria.

Cyanotoxins are produced by cyanobacteria of the genera *Anabaena* and *Nostoc* among others and include some of the most powerful natural poisons that target the nervous system (neurotoxins: BMAA, saxitoxin and anatoxin-a), the liver (hepatotoxins: microcystins and nodularins), protein synthesis and DNA modification (cylindrospermopsin) and the skin (dermatotoxins; nodularin). Cyanotoxins are alkaloids (anatoxin-a, saxitoxin, cylindrospermopsin) or peptides (pentapeptide nodularin or the heptapeptide microcystin) [18–21]. Upon their release in water, they are ingested by zooplankton and animals, or absorbed by phytoplankton and plants that can have acute or chronic effects when eaten by humans. This is a global health issue owing to bioconcentration and bioaccumulation in the food chain and poisoning through ingestion of contaminated food, so that cyanotoxins are now widely analyzed and studied to determine their effects on plants and animals [22]. For example, the World Health Organization (WHO) recommends a value of 1 µg/L for microcystin-LR in drinking water [23].

BMAA (β-N-methylamino-L-alanine) is a non-proteinogenic amino acid is produced by free-living cyanobacteria in marine, freshwater, and terrestrial environments [24,25]. It has been detected in plants with endosymbiotic cyanobacteria including lichens, hornworts, the leaf petioles of the tropical flowering plant *Gunnera*, and the cycad *Cycas circinalis* [24–26] and linked to the amyotrophic

lateral sclerosis/parkinson-dementia complex (ALS/PDC) detected among the Chamorro people living on the Pacific island of Guam [24,25]. BMAA, like other cyanotoxins, can be biomagnified in seafood eaten by people, including fish [27,28], shrimps [29], mussels, oysters, and crabs [30]. BMAA can also be synthesized by eukaryotes such as diatoms [31] and dinoflagellates [32] which are food sources for crustaceans, fish and shellfish [33].

These observations raise the question of whether eating *Azolla* may be harmful to humans due to the possible production of BMAA and other cyanotoxins by *N. azollae*. Unlike free-living *Anabaena* and *Nostoc*, the loss or conversion to pseudogenes of genes involved in cyanotoxin and/or BMAA production may have occurred in *N. azollae* because they were no longer needed by the permanently enclosed cyanobiont. The following analyses were therefore undertaken on all seven extant *Azolla* species and their cyanobionts to determine if they can be safely eaten by people:

- The presence of genes coding for microcystin, nodularin, cylindrospermopsin and saxitoxin.
- The presence of the anatoxin-a/homoanatoxin-a gene cluster by bioinformatic tools.
- The presence of BMAA.

The seven examined species of *Azolla* are *A. caroliniana*, *A. filiculoides*, *A. mexicana*, *A. microphylla*, *A. nilotica*, *A. rubra* and *A. pinnata* including its two subspecies *A. pinnata* subsp. *pinnata* and *A. pinnata* subsp. *imbricata*. Table 1 lists the 48 accessions that provided the *Azolla* species and subspecies used in this study.

Table 1. List of *Azolla* accessions from worldwide countries.

Accession ^a	Species name	Origin and harvest year	Source ^b /collector
PI1 ^{*s}	<i>A. pinnata</i> subsp. <i>imbricata</i>	Philippines, Sto Domingo, Albay, 1975	IRRI
PI2		Malaysia, Bumbong Lima, Butterworth, 1977	IRRI
PI23		India, Cuttack, Orissa, 1978	CRRRI
PI68		Sri Lanka, Tissa, 1984	S. Kulasooriya
PI102		Japan, Okinawa, 1987	O. Mochida
PI503		Australia, Murdoch, 1978	M. Dilworth
PI531		Indonesia, Bali, 1983	-
PI540		China, Putian, 1989	C. van Hove
FI1001 [*]	<i>A. filiculoides</i>	East Germany (ex- GDR), 1979	IB China
FI1008		USA, Cranmore Road, Sutter Co., California, 1981	D. Rains
FI1010		Peru, PUFFI, Lima, 1982	CIAT
FI1042		Brazil, Parana, 1987	I. Watanabe
FI1052		South of France, North of Lyon, 1989	P. Roger
FI1090		Japan, Tanabe-cho, 1992	S. Kitoh
FI1501		Belgian, Harchies, 1987	A. Lawalree
FI1505		South Africa, Verwoerd dam, 1987	D. Toerien
FI1507 ^s		Colombia, Zipaquira, 1987	Y. Lopez
FI1522		Switzerland, Zurich Botanical Garden, 1987	-
FI-BGLU		Botanical Garden of Lisbon University, 2009	A.L. Pereira
FI-BGM		Botanical Garden of Madeira, Funchal, 2010	C. Lobo
ME2001 [*]	<i>A. mexicana</i>	USA, Graylodge, California, 1978	D. Rains
ME2008		Colombia, CIAT, Cali, 1982	CIAT
ME2011		Japan, Osaka, 1984	T. Lumpkin
ME2026 ^s		Brazil, Solimoes river, Pacencia Island, Iranduba, Amazonas (BLCC 18), 1984	T. Lumpkin
CA3001 ^{*s}	<i>A. caroliniana</i>	USA, Ohio, 1978	D. Rains
CA3017		Brazil, Rio Grande do Sul, 1987	I. Watanabe
CA3502		Egypt, Moshthohr University, 1987	C. Myttenaere
CA3507		Suriname, Boxel, 1987	H. Lardinois

CA3513		Zimbabwe, Causeway Botanical Garden, 1987	T. Muller
CA3524		Holland, 1987	E. Ohoto
CA3525		Ruanda, Cyili Rice Research Center, 1987	C. van Hove
MI4018 ^a	<i>A. microphylla</i>	Paraguay, 1981	D. Rains
MI4021 ^s		Equator, Santa Cruz Island, Galapagos, 1982	T. Lumpkin
MI4028		Philippines, hybrid (MI4018xFI1001), 1985	Do Van Cat
MI4054		Brazil, Baía, 1987	I. Watanabe
MI4510		Philippines, Los Baños, IRRI, 1987	C. van Hove
NI5001 ^{*s}	<i>A. nilotica</i>	Sudan, Kosti, 1982	T. Lumpkin
NI5002 [#]		Sudan, Kosti, 1989	T. Lumpkin
NI5501		Burundi, Bujumbura, 1987	J. Bouharmont
RU6010 [*]	<i>A. rubra</i>	New Zealand, Nouville, 1986	C. van Hove
RU6502		Australia, Victoria (37.40S-144.40E), 1985	-
RU6503		New Zealand, between Lumdsen and Kingston, 1986	C. van Hove
PP7001 ^{*s}	<i>A. pinnata</i> subsp. <i>pinnata</i>	Australia, Kakadu Northern Park Northern Territory, 1982	Yatazawa
PP7506		Sierra Leone, 1982	C Dixon
PP7509		Nigeria, Moor plantation, 1987	C. van Hove
PP7511		Guinea-Bissau, Contuboel, 1987	H. Diara
PP7512		Zaire, Kisantu, 1987	B. Bruyneel
PP7546		Madagascar, Antsahavory, East zone, 1991	C. van Hove

^aAccession numbers were listed according to IRRI code number except for Portuguese accessions (FI-BGLU and FI-BGM); - Unknown collector or germplasm source. ^bCIAT-International Centre for Tropical Agriculture, Colombia, CRR- Cyili Rice Research Center, IB China-Institute of Botany, Academia Sinica, Beijing, China, IRRI-International Rice Research Institute. [#] *N. azollae* was not isolated from this *Azolla* accession. ^{*}BMAA extracted from *Azolla* and *Nostoc azollae* (isolated from *Azolla*, see 4.2.) with method 1 (see 4.3.1.). ^sBMAA extracted from *Azolla* accessions with method 2 (see 4.3.2.).

2. Results

2.1. The Cyanotoxins Microcystin, Nodularin, Saxitoxin, Cylindrospermopsin and Anatoxin-a

The PCR amplification of 12 genes that encode for four cyanotoxins (cylindrospermopsin, nodularin, saxitoxin and microcystin) was determined for all seven *Azolla* species and two *A. pinnata* subspecies from 48 *Azolla* accessions listed in Table 1. Global distribution of the accession countries is shown in Supplementary Material Figure S25. The results indicate that cyanotoxin gene amplification on *Azolla* accessions were negative when matched with positive and negative controls for 12 genes: *cyl*, *mcy A*, *mcy B*, *mcy B* domain A, *mcy C*, *mcy C* domain A, *mcy D* domain ACP, *mcy D* domain KS, *mcyE/ndaF*, *mcy E* domain GSA-AMT, *mcy G* domain CM, *sxt*.

The results also showed that same 12 genes in *N. azollae* isolated from the 47 *Azolla* accessions were not amplified compared with positive and negative controls. This indicates that both *Azolla* and *N. azollae* do not have genes that biosynthesize those cyanotoxins. Photographs of all gels from the PCR amplifications are shown in Supplementary Material Figures S1 to S24.

The BLAST search for the anatoxin-a and homoanatoxin-a gene cluster showed a query cover of only 3% and percent identity of 73.04%. Most of these alignments were partial segments comprising less than 250 bp of the *anaH* transposase gene with identities around 70%. The aligned genes in *N. azollae* are only associated with pseudogenes and not with protein coding genes. All other alignments were partial, and none included any of the whole genes associated with the anatoxin-a biosynthesis gene cluster.

2.2. Detection of BMAA (β -N-methylamino-L-alanine)

The detection of the non-proteinogenic amino acid BMAA by LC-MS/MS with method 1 on all seven *Azolla* species (*A. caroliniana*, *A. filiculoides*, *A. microphylla*, *A. mexicana*, *A. nilotica*, *A. rubra* and

the two subspecies of *A. pinnata*) showed that the BMAA was absent from both *Azolla* and its cyanobiont *N. azollae* since there is not found the retention time for BMAA and the isomer 2,4-DAB (2,4-diaminobutyric acid) when compared with their standards which are not shown).

Re-analysis of six of the *Azolla* species (*A. rubra* that was not re-analyzed) using method 2, in which the samples were derivatized, corroborated the results obtained with method 1. This indicates that the *Azolla* species shown in Figure 1 did not show the retention times for BMAA (RT=13.92 min) and the two isomers 2,4-DAB (RT=14.93 min), and AEG (N-(2-aminoethyl)-glycine) (RT=13.22 min) when compared with their standards (Figure 2).

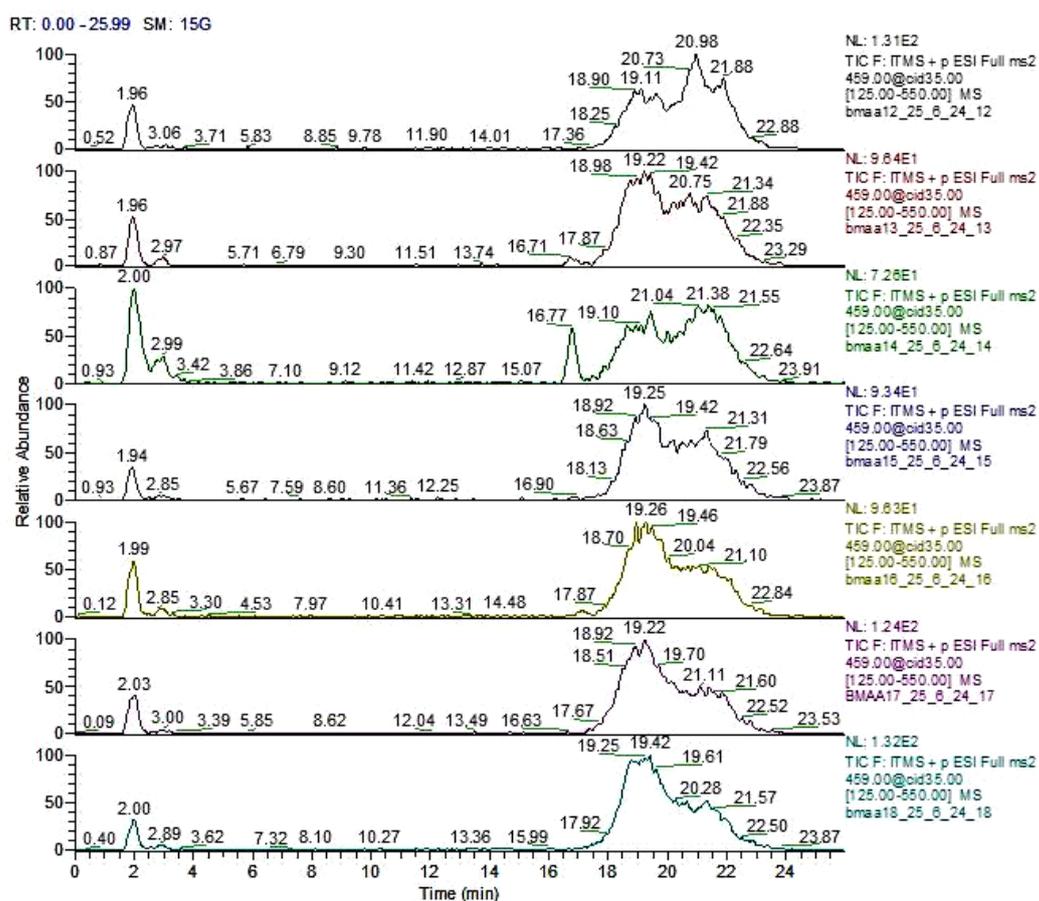


Figure 1. Total ion chromatogram of six *Azolla* species for the detection of BMAA with method 2. From top to bottom: *A. caroliniana* (CA 3001), *A. filiculoides* (FI 1507), *A. pinnata* subsp. *pinnata* (PI 7001), *A. nilotica* (NI 5001), *A. mexicana* (ME 2026), *A. microphylla* (MI 4021), *A. pinnata* subsp. *imbricata* (PI 1).

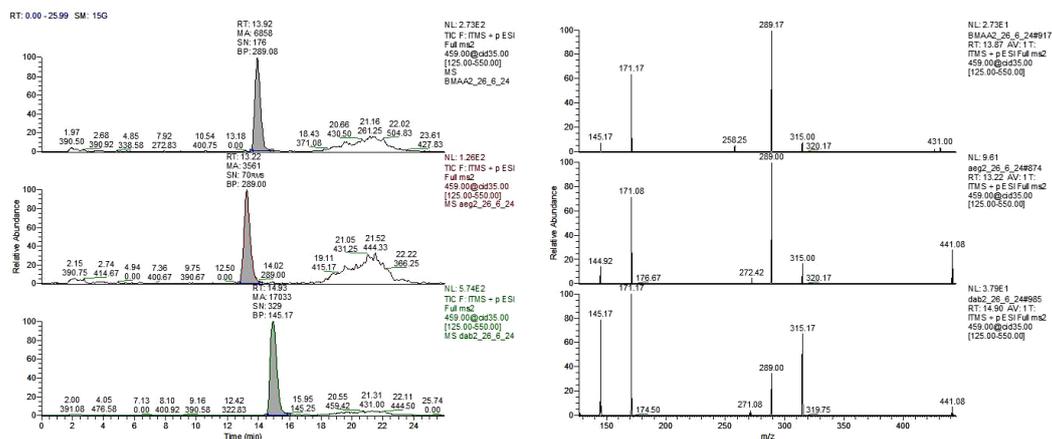


Figure 2. Total ion chromatogram (left) and CID spectra (right) of derivatized standards at 1 ppm. BMAA (top, RT=13.92 min), AEG (middle, RT=13.22 min), and 2,4-DAB (bottom, RT=14.93 min).

3. Discussion

Most genera of free-living cyanobacteria synthesize cyanotoxins and include cyanobacteria that have temporary symbiosis with some plants, so that the host plant has the potential to assimilate and bioaccumulate the cyanotoxins, discussed above. There are few published studies documenting genetic and chromatographic detection of cyanotoxins in cyanobionts. Cyanobacteria from lichens have been analysed and contain genes that encode nodularin and microcystin, and can translate the peptides nodularin and microcystins [34–36]. The synthesis of those two cyanotoxins may be linked to the temperature and humidity in which the lichens grow and may be important for the maintenance of lichens in diverse ecological habitats [37].

Unlike lichens, the fern *Azolla* has a permanent symbiosis with the cyanobacteria *N. azollae* giving this symbiosis a unique evolution pattern and, ultimately, the loss of genes by the cyanobiont [4–7]. Our genetic analyses show, for the first time, that all seven *Azolla* species and their cyanobiont, *N. azollae*, do not possess genes associated with the synthesis of microcystin, nodularin, saxitoxin, cylindrospermopsin, anatoxin-a, and homoanatoxin-a. The biosynthetic pathways of microcystins [38] and anatoxin-a/homoanatoxin-a [39] are a multi-step process that requires several genes to synthesize both cyanotoxins. All genes associated with microcystin synthesis were, therefore, amplified by specific primers with *Azolla* and *N. azollae* DNA, and the complete anatoxin-a gene cluster was BLAST searched against the *N. azollae* genome. There were no matches, supporting the model that *N. azollae* lost the ability to synthesise microcystin, nodularin, saxitoxin, cylindrospermopsin, anatoxin-a, and homoanatoxin-a due to downregulation of cyanotoxin biosynthesis genes or loss of the genes during the co-evolution of *Azolla* and *N. azollae*.

BMAA was isolated in 1967 from seeds of *Cycas circinalis* (cycad) [24–26] and identified as the primary cause of amyotrophic lateral sclerosis/parkinson-dementia complex (ALS/PDC) in the Chamorro people on the Pacific island of Guam [40,41], with the high levels of the neurotoxin resulting from biomagnification through the food chain [27,28,33,42,43]. BMAA is a cyanotoxin that can cross the blood-brain barrier where it forms a reservoir [44] and can be inserted into proteins instead of the amino acid L-serine, causing protein misfolding and aggregation [45–47]. BMAA can induce changes in the expression of genes in brain cells and thus resulting in a wide range of other neurodegenerative disorders [19]. Alzheimer's, Parkinson's and other neurological diseases including amyotrophic lateral sclerosis (ALS), progressive supranuclear palsy (PSP) and dementia with Lewy bodies (DLB) [48] may therefore be partially caused or facilitated by BMAA. However, the gene/genes for the codification of BMAA are not known in any cyanobacteria and plant, so that their presence can only be detected by analytical methods, including those used in this study.

BMAA was detected in plant-cyanobacteria symbiosis such as hornworts, liverwort, lichens, cycads and *Gunnera* [24] and also in *A. filiculoides* with 2 µg/g [42]. Some analytical methods to detect this non-proteinogenic molecule can result in erroneous interpretations due to structural isomers DAB (2,4-diaminobutyric) and AEG (N-(2-aminoethyl)-glycine which can co-elute and be misidentified as BMAA [49,50]. For the present study, two methods were therefore used to detect BMAA, DAB and AEG, and did not detect BMAA, AEG, or DAB in any of the analyzed *Azolla* and *N. azollae*. These data indicate that the previous reported detection of 2 µg/g BMAA in *Azolla* [42] is incorrect.

Harmful algal blooms (HABs) of other cyanobacteria species also release cyanotoxins upon cell necrosis. The uptake and bioaccumulation of cyanotoxins from irrigated water for crop and non-crop therefore also needs to be evaluated to determine if *Azolla* species may bioaccumulate cyanotoxins. Previous studies show that *A. filiculoides* does not uptake or bioaccumulate microcystin [51] or cylindrospermopsin [52], confirming that *Azolla* can be safely eaten.

4. Materials and Methods

4.1. *Azolla* Accessions and Culturing

The seven *Azolla* species including two *A. pinnata* subspecies from the germplasm collection at IRRI (International Rice Research Institute) and two *A. filiculoides* accessions from Portugal (FI-BGLU and FI-BGM) were used to detect the cyanotoxin genes of microcystin, nodularin, cylindrospermospin and saxitoxin by PCR, and BMAA by LC-MS/MS (Table 1.) The 48 *Azolla* accessions have a global distribution from 33 countries (Supplementary Material, Figure S25). The *Azolla* species were cultured in Hoagland medium (H-40), pH 6.1-6.2, at controlled temperature, photoperiod, and light intensity [53]. The biomass was collected, washed in distilled water, frozen at -80°C, lyophilized, and then weighed.

4.2. Isolation of *Nostoc azollae* from *Azolla* accessions

Nostoc azollae cyanobionts were isolated from the dorsal foliar cavities of 48 *Azolla* accessions (Table 1) using the gentle roller method [54,55] with the following modifications. Roots were cut-off and sporophytes were disinfected in aqueous sodium hypochlorite (1 ml NaClO:10 ml distilled water, v:v) for 20 minutes, followed by three washes in ultrapure water (Millipore, Madrid, Spain). Sporophytes were sectioned and squeezed with a roller to separate the cyanobiont from *Azolla* cavities. The extract (*Azolla*+water+*N. azollae*) was centrifuged twice at 3000 g for 3 minutes to settle fern debris. The recovered supernatant with *N. azollae* filaments was centrifuged twice at 1000 g for 1 minute to free cyanobionts from the cellular debris. The recovered dark-green pellet was centrifuged at 11000 g for 10 minutes, stored at -20°C, frozen at -80°C, lyophilized and weighted.

4.3. Detection and Analysis of BMAA (β -N-Methylamino-L-Alanine)

4.3.1. Method 1

The methodology, including reagents and materials, described by Baptista et al. (2015) [56] was used., with extraction of BMAA and quantification by LC-MS/MS using validated analytical methods [50,56]. Lyophilized *Azolla* and *N. azollae* (10 mg each sample) were acid-digested in 6 M HCl at 90°C for 20 minutes, using a high-pressure microwave system (Milestone-Ethos 1). After evaporation with nitrogen, 20 mM of HCl was added to samples and filtered (0.22 μ m Millipore).

Analyses of BMAA by LC-MS/MS were performed in a Thermo LCQ Fleet Ion Trap LC/MSn system (Thermo Scientific) using a 2.1 \times 100 mm, 5- μ m diameter ZIC-HILIC column (SeQuant) and a 14 \times 1 mm, 5- μ m guard column (SeQuant). The mobile phase was acetonitrile (0.1% formic acid) and deionized water (0.1% formic acid). A linear gradient of 90% acetonitrile for 20 minutes was followed by 60% acetonitrile for 15 minutes and 90% acetonitrile for 5 minutes. The flow rate was 0.5 ml min⁻¹, injection volume was 10 μ l, column temperature at 40°C and the positive mode on the electrospray ionization (ESI). Nitrogen was the sheath gas at a rate of 45 (unitless), and auxiliary gas at a rate of 20 (unitless). The capillary temperature was held at 250°C. Mass-to-charge ratio (m/z) scan was performed from 50 to 150 and the ion m/z 119 to assess 2,4-DAB (2,4-diaminobutyric acid) was monitored. The occurrence of the product ions m/z 102, 88 and 76 was verified at collision energy of 14 V for the presence of BMAA.

4.3.2. Method 2

Method 2 followed that described by Pravadali-Cekic S. et al. (2023) [49] with some modifications for the amount of starting material, chromatographic column, eluents and mode of mass detection. Lyophilized *Azolla* biomass, (100 mg) was dissolved in 3 ml of trichloroacetic acid (TCA) 10% (v/v) and sonicated on ice (5 min, 70% amplitude, 20Hz), followed by overnight precipitation at 4°C. The mixture was then centrifuged (5000 g, 15 min, 4°C), the supernatant reserved and the pellet submitted to a second extraction cycle. The third extraction step used 10% TCA/acetone. The pellet as the bound fraction was transferred to a glass vial with acetone (100 %), centrifuged, and the supernatant added to the free fraction. The pooled free fraction was then evaporated to dry in a SpeedVac and kept at -80°C. Pellets were also dried using the SpeedVac and acid hydrolysis by adding 3 ml of 6 M HCl overnight at 110°C. The hydrolyzed pellet was re-suspended in 1 ml ultrapure water and added to the free fraction. Samples were then filtered through a This was carried out with

a 20 μ l standard mix solution or sample extract, 20 μ l of derivatizing reagent, and 60 μ l of borate buffer. Following AccQ-Tag Ultra Derivatization Kit in accordance with the manufacturer's guidelines, the mixture was vortexed for several seconds and placed in a thermocycler at 55°C for 10 minutes. The final extract was then transferred to a 1.5mL vial for LC/MS/MS analysis.

Samples were injected in a Liquid Chromatograph Thermo Finnigan Surveyor HPLC System (Thermo Scientific, MA, USA), coupled with a Mass Spectrometry LCQ Fleet™ Ion Trap Mass Spectrometer (Thermo Scientific, MA, USA). Xcalibur™ version 2. Mass Spectrometer Tune Method parameters optimization was used for data acquisition and processing using direct injection of BMMA and co-occurring isomers in a solution of 1 ppm in LCMS grade water (Table 2). The Mass Spectrometer operated in electrospray positive polarity mode using Collision Ionisation Dissociation (CID) corresponding to the $[M+H]^+$ BMMA, AEG (N-(2-aminoethyl)-glycine), and 2,4-DAB molecules ion precursors and respective diagnostic fragments. The spray voltage was maintained at 3.5 kV, capillary temperature at 350°C, and capillary voltage at 20 kV and tube lens at 120 kV. Nitrogen was used as the sheath and auxiliary gas, with collision energy at 20 eV in Collision Induced Dissociation Mode. Separation was achieved on an ACE Excel C18 (50 \times 2.1 mm I.D., 1.7 μ m, Batch: V17-1253, Avantor® ACE®, VWR, PT) at 18°C, with a flow rate of 0.3 ml/min injected at a volume of 10 μ l in a no-waste mode. The eluents used were methanol (A) and water (B), both acidified with formic acid at 0.1% (v/v). The gradient program started at 13% A, increasing to 90% A in 20 minutes, turning back to initial conditions in 5 minutes, equilibrating for an additional 10 minutes with 20% A. See Table 2 for the chromatographic and mass parameters.

Table 2. Chromatographic and Mass parameters for the BMMA and the isomers AEG and DAB detection.

Target	Retention time (min)	Derivatized Ion precursor (m/z)	CID Fragments (m/z)	CID Collision Energy (V)	Calibration: Curve and Linear interval (μ g/L)	LOD (μ g/L)	LOQ (μ g/L)
BMAA	13.92 \pm 0.08	459.00	320,315,289,258,171,14 5	35	y=1377.4x-2154.1 r ² =0.9947 3-250	7.99	24.2
AEG	13.22 \pm 0.11	459.00	320,315,289,272,171,14 5	35	y=889.91x+4972.2 r ² =0.9993 3-150	57.4	174
2,4-DAB	14.93 \pm 0.15	459.00	320,315,289,271,171,14 5	35	y=2785x+5538.1 r ² =0.9934 7-250	70	212

LOD - limit of detection; LOQ - limit of quantification; CID - collision induced dissociation.

4.4. Cyanotoxin Genes at *Azolla* Accessions and *Nostoc Azollae*

4.4.1. DNA Extraction

DNA from *N. azollae* was extracted with PureLink® Genomic DNA MiniKit (Invitrogen, Carlsbad, California, USA) and DNA from *Azolla* accessions was extracted with Genomic DNA from Plant NucleoSpin® Plant II (Macherey-Nagel, Düren, Germany) according to the manufacturer instructions. DNA was stored at -20°C. The DNA was quantified in a Qubit fluorometer (Invitrogen) using the Quant-iT® dsDNA HS assay following the manufacturer instructions. A working DNA concentration of 0.1 μ g/ μ l was made with sterile ultrapure water, and saxitoxin, nodularin, and microcystin were assessed by specific primers (Table 3). A Biometra TProfessional (Goettingen) thermocycler was used for PCR amplification using the conditions listed in Table 4 for each gene, with a hold at 4°C for all the programs. Each 20 μ l reaction contained 1 μ l of 0.5 μ M of each primer (Invitrogen), 2 μ l of 0.1 μ g/ μ l DNA, 9 μ l Supreme NZYtaq 2x Green Master Mix (NZYTech) and 7 μ l of ultrapure sterile water. Negative (with sterile ultrapure water) and positive (*Microcystis aeruginosa* LEGE91094 for microcystin and microcystin/nodularin genes, *Aphanizomenon ovalisporum* for

cylindrospermopsin gene and *Aphanizomenon gracillaris* LMECYA 40 from INSA for saxitoxin gene) controls were included. The amplification products were separated in 1.5% agarose gel electrophoresis running in TAE 1x at 150 V, 25-30 min and stained with 0.2 µg/ml ethidium bromide (BioRad). The 1 Kb Plus DNA ladder (Invitrogen) was used as molecular size marker.

Table 3. Primers used to amplify cyanotoxic genes in *Azolla* and *N. azollae* DNA.

Gene	Primer	Sequence primer (5'→3')	Size (bp)	Reference
Saxitoxin (<i>sxt</i>)	SXT683F	GGATCTCAAACATGATCCCA	195	Lopes et al. 2012
	SXT877R	GCCAAACGCAGTACCACTT		
Cylindrospermopsin (<i>cyl</i>) (poliketide synthase)	K18F	CCTCGCACATAGCCATTGTC	422	Schembri et al. 2001
	M4R	GAAGCTCTGGAATCCGGTAA		
Cylindrospermopsin (<i>cyl</i>) (peptide synthase)	M13	GGCAAATTGTGATAGCCACGAGC	597	Fergusson 2003
	M14	GATGGAACATCGCTCACTGGTG		Schembri et al. 2001
Microcystin/Nodularin synthetase (<i>mcyE/ndaF</i>)	HepF	TTTGGGGTAACTTTTTGGCCAT AGTC	472	Jungblut 2006
	HepR	AATTCTTGAGGCTGTAAATCGGG TTT		
Microcystin synthetase (<i>mcy A</i>)	<i>mcyA</i> -Cd1F	AAAATTTAAAAGCCGTATCAAA	297	Hisbergues et al. 2003
	<i>mcyA</i> -Cd1R	AAAAGTGTTTTATTAGCGGCTCAT		
Microcystin synthetase (<i>mcy B</i>)	2959F	TGGGAAGATGTTCCTCAGGTATCC AA	350	Nonneman & Zimba 2002
	3278R	AGAGTGGAAACAATATGATAAG CTAC		
Microcystin (<i>mcy C</i>)	FAA	CTATGTTATTTATACATCAGG	758	Neilan 1999
	RAA	CTCAGCTTAACTTGATTATC		
Microcystin (<i>mcy B</i> , domain A)	2156F	ATCACTTCAATCTAACGACT	955	Mikalsen 2003
	3111R	GTTGCTGCTGTAAGAAA		
Microcystin (<i>mcy C</i> , domain A)	PSCF1	GCAACATCCCAAGAGCAAAG	674	Ouahid 2005
	PSCR1	CCGACAACATCACAAGGC		
Microcystin (<i>mcy D</i> , domain ACP)	PKDF1	GACGCTCAAATGATGAAACT	647	Ouahid 2005
	PKDR1	GCAACCGATAAAAACCTCCC		
Microcystin (<i>mcy D</i> , domain KS)	PKDF2	AGTTATTCTCCTCAAGCC	859	Ouahid 2005
	PKDR2	CATTCGTTCCACTAAATCC		
Microcystin (<i>mcy E</i> , domain GSA-AMT)	PKEF1	CGCAAACCCGATTTACAG	755	Ouahid 2005
	PKER1	CCCCTACCATCTTCATCTTC		
Microcystin (<i>mcy G</i> , domain CM)	PKGF1	ACTCTCAAGTTATCCTCCCTC	425	Ouahid 2005
	PKGR1	AATCGCTAAAACGCCACC		

Table 4. Amplification conditions for the cyanotoxic genes in *Azolla* and *N. azollae* DNA.

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Reference
<i>sxt</i>	94°C; 3 min	94°C; 10 s	35 cycles 52°C; 20 s	72°C; 1 min	72°C; 7 min	Lopes et al. 2012
<i>cyl</i>	94°C; 10 min	94°C; 30 s	30 cycles 55°C; 30 s	72°C; 7 min	72°C; 7 min	Fergusson 2003
<i>mcyE/ndaF</i>	92°C; 2 min		35 cycles			

		92°C; 20 s	56°C; 30 s	72°C; 1 min	72°C; 5 min	Jungblut 2006
<i>mcy A</i>	95°C; 2 min	35 cycles			72°C; 7 min	Hisbergues et al. 2003
		95°C; 90 s	56°C; 30 s	72°C; 50 s		
<i>mcy B</i>	94°C; 2 min	35 cycles			72°C; 5 min	Nonneman 2002
		94°C; 30 s	59°C; 45 s	72°C; 1 min		
<i>mcy C</i>	94°C; 2 min	35 cycles			72°C; 7 min	Neilan 1999
		94°C; 10 s	50°C; 20 s	72°C; 1 min		
<i>mcy B</i> , domain A	94°C; 4 min	30 cycles			72°C; 7 min	Mikalsen 2003
		95°C; 30 s	52°C; 30 s	72°C; 1 min		
<i>mcy C</i> , domain A	94°C; 5 min	35 cycles			72°C; 7 min	Ouahid 2005
		95°C; 1 min	52°C; 30 s	72°C; 1 min		
<i>mcy D</i> , domain ACP	94°C; 5 min	35 cycles			72°C; 7 min	Ouahid 2005
		95°C; 1 min	52°C; 30 s	72°C; 1 min		
<i>mcy D</i> , domain KS	94°C; 5 min	35 cycles			72°C; 7 min	Ouahid 2005
		95°C; 1 min	52°C; 30 s	72°C; 1 min		
<i>mcy E</i> , domain GST-AMT	94°C; 5 min	35 cycles			72°C; 7 min	Ouahid 2005
		95°C; 1 min	52°C; 30 s	72°C; 1 min		
<i>mcy G</i> , domain CM	94°C; 5 min	35 cycles			72°C; 7 min	Ouahid 2005
		95°C; 1 min	52°C; 30 s	72°C; 1 min		

4.4.3. BLAST of Anatoxin-a Genes against *Nostoc azollae*

To determine if *N. azollae* produces anatoxin-a, a nucleotide BLAST (BLASTN) search for anatoxin-a coding genes was performed. Since the anatoxin-a gene cluster was discovered after the PCR and gel analysis of the other cyanotoxins performed in this study, analysis of its presence was made separately through BLAST rather than as the query sequence [57]. This was a 34682 bp sequence encoding for proteins associated with the biosynthesis of these toxins. The nucleotide query was applied to the full genome of *Nostoc azollae* 0708 (taxid: 551115). Matches with E-values from the BLASTN less than 0.01 were investigated and analyzed.

5. Conclusions

Our LC-MS/MS results show the *Azolla-Nostoc azollae* superorganism does not contain BMAA or their isomers DAB and AEG, and that *Azolla* and *N. azollae* do not synthesize other common cyanotoxins, indicating that *Azolla* is a nutritious food that can be safely eaten.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. **Supplementary Materials 1, Figures S1-S24:** Agarose gel from the PCR amplification of genes in *Azolla* and *N. azollae*. **Figure S25:** Map showing the countries of *Azolla* accessions origin used in the present study. **Figure S1:** Agarose gel from the PCR amplification of the gene microcystin/nodularin synthetase for nodularin in 48 *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder; **Figure S2:** Agarose gel from the PCR amplification of the gene saxitoxin in 48 *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*A. gracillaris* LMECYA 40), C-: negative control, 1st line is Ladder. **Figure S3:** Agarose gel from the multiplex PCR amplification of the genes poliketide synthase and peptide synthase for cylindrospermopsin in 48 *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*A. ovalisporum*), C-: negative control, 1st line is Ladder. **Figure S4:** Agarose gel from the PCR amplification of the gene microcystin synthetase (*mcy A*) for

microcystin in 48 *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S5:** Agarose gel from the PCR amplification of the gene microcystin synthetase (mcy B) for microcystin in 48 *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S6:** Agarose gel from the PCR amplification of the gene microcystin C for microcystin in 48 *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S7:** Agarose gel from the PCR amplification of the gene microcystin B A-domain for microcystin in 48 *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S8:** Agarose gel from the PCR amplification of the gene microcystin C A-domain for microcystin in 48 *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S9:** Agarose gel from the PCR amplification of the gene microcystin D ACP-domain for microcystin in 48 *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S10:** Agarose gel from the PCR amplification of the gene microcystin D KS-domain for microcystin in 48 *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S11:** Agarose gel from the PCR amplification of the gene microcystin E GSA-AMT-domain for microcystin in 48 *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S12:** Agarose gel from the PCR amplification of the gene microcystin G CM-domain for microcystin in 48 *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S13:** Agarose gel from the PCR amplification of the gene microcystin/nodularin synthetase for nodularin in 47 *Nostoc azollae* isolated from *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S14:** Agarose gel from the PCR amplification of the gene saxitoxin in 47 *Nostoc azollae* isolated from *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*A. gracillaris* LMEDIA 40), C-: negative control, 1st line is Ladder. **Figure S15:** Agarose gel from the multiplex PCR amplification of the genes poliketide synthase and peptide synthase for cylindrospermopsin in 47 *Nostoc azollae* isolated from *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*A. ovalisporum*), C-: negative control, 1st line is Ladder. **Figure S16:** Agarose gel from the PCR amplification of the gene microcystin synthetase (mcy A) for microcystin in 47 *Nostoc azollae* isolated from *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S17:** Agarose gel from the PCR amplification of the gene microcystin synthetase (mcy B) for microcystin in 47 *Nostoc azollae* isolated from *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S18:** Agarose gel from the PCR amplification of the gene microcystin C for microcystin in 47 *Nostoc azollae* isolated from *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S19:** Agarose gel from the PCR amplification of the gene microcystin B A-domain for microcystin in 47 *Nostoc azollae* isolated from *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S20:** Agarose gel from the PCR amplification of the gene microcystin C A-domain for microcystin in 47 *Nostoc azollae* isolated from *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S21:** Agarose gel from the PCR amplification of the gene microcystin D ACP-domain for microcystin in 47 *Nostoc azollae* isolated from *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S22:** Agarose gel from the PCR amplification of the gene microcystin D KS-domain for microcystin in 47 *Nostoc azollae* isolated from *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S23:** Agarose gel from the PCR amplification of the gene microcystin E GSA-AMT-domain for microcystin in 47 *Nostoc azollae* isolated from *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S24:** Agarose gel from the PCR amplification of the gene microcystin G CM-domain for microcystin in 47 *Nostoc azollae* isolated from *Azolla* accessions (see Table 1 from the manuscript). C+ -: positive control (*M. aeruginosa* LEGE 91094), C-: negative control, 1st line is Ladder. **Figure S25:** Map showing the countries of *Azolla* accessions origin used in the present study. Legend: a – *A. pinnata* subsp. *imbricata*; b – *A. filiculoides*; c – *A. mexicana*; d – *A. caroliniana*; e – *A. microphylla*; f – *A. nilotica*; g – *A. rubra*; h – *A. pinnata* subsp. *Pinnata*. **Supplementary Materials 2:** Sequence alignment from the BLASTN query between the Anatoxin-a gene cluster and the *Nostoc azollae* genome.

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