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ORIGINAL ARTICLE

The non-protein amino acid *β*-*N*-methylamino-L-alanine in Portuguese cyanobacterial isolates

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Abstract The tailor made amino acid *β*-*N*-methyl-amino-L-alanine (BMAA) is a neurotoxin produced by cyanobacteria. It has been associated with certain forms of progressive neurodegenerative disease, including sporadic Amyotrophic Lateral Sclerosis and Alzheimer's disease. Some different reports of BMAA in cyanobacterial blooms from lakes, reservoirs, and other water resources have been made by different investigators. We here report the detection of BMAA of both free and protein-bound produced by cyanobacteria, belonging to the Chroococcales, Oscillatoriales and Nostocales ordered. We use a rapid and sensitive HPLC-FD method that utilizes methanol elution and the Waters AQC Tag chemistry. On other hand, we have used three different assay procedures for BMAA extraction from cyanobacteria: Trichloroacetic acid (TCA), Methanol/Acetone and hydrochloric acid (HCl). All assays let successfully detect BMAA in all cyanobacteria samples analyzed. Nevertheless, with TCA and HCl extraction procedures the highest BMAA values, for free as well as protein-bound BMAA were detected. BMAA content could not be related to the taxonomy of the isolates or to their geographical origin, and no correlation between free and protein-bound BMAA concentrations were observed within or between taxonomic groups. These data offer confirmation of the taxonomic and geographic ubiquity of BMAA from naturally occurring populations of cyanobacteria, for the first time reported for estuaries.

Keywords AQC · BMAA · Cyanobacteria · Neurotoxin · HPLC-FD · Estuaries

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Introduction

It has been suggested that the tailor made amino acid *β*-N-methylamino-L-alanine (BMAA) (Soloshonok et al. 1999) is linked to neurodegenerative diseases (Liu et al. 2009). BMAA was originally isolated from the seeds of the cycad tree (*Cycas* sp.) in 1967, in Guam (Vega and Bell 1967). Biomagnification of BMAA through trophic chains was first proposed for the Guam ecosystem and showed the presence of BMAA from the endosymbiotic *Nostoc* sp. (on the coralloid roots of *Cycas*) (Cox et al. 2003). The Chamorro people of Guam have been afflicted with a complex of neurodegenerative diseases now known as Amyotrophic Lateral Sclerosis-Parkinsonism Dementia Complex (ALS/PDC) (Cox and Sacks 2002; Cox et al. 2003; Murch et al. 2004a, b; Banack et al. 2006; Papapetropoulos 2007). The statistical correlation (Reed et al. 1987) of risk for ALS/PDC with the consumption of a traditional Chamorro diet implicated cycads as a possible source of neurotoxin.

This biomagnification hypothesis was challenged by the fact that exposing animals to a BMAA-enriched diet did not produce toxic effects or produced symptoms of acute toxicity, which was inconsistent with the model of a

progressive neurodegenerative disease (Karamyan and Speth 2008). However, while an animal model for BMAA-induced ALS was lacking, the hypothesis was not discarded due to the verified in vitro neurotoxicity of BMAA (Liu et al. 2009; Lobner et al. 2007; Rao et al. 2006).

The specificities of the Guam ecosystem, in which the biomagnification of the neurotoxin was via the ingestion of an uncommon dietary item deemed impossible the extrapolation of the biomagnification mechanism to other populations. But the recognition that the endosymbiotic *Nostoc* sp. could produce BMAA, when cultured after isolation from the roots of *Cycas* sp., led to the detection that a large number of cyanobacteria strains could produce BMAA, either as symbionts or as free-living species in marine, brackish- or freshwater biota (Cox et al. 2005; Esterhuizen and Downing 2008; Metcalf et al. 2008).

Recently, biomagnification of BMAA in food webs of the North Atlantic has been shown, from cyanobacteria, to zooplankton, to invertebrates, and to vertebrates (fish) with increasing BMAA concentration within higher trophic levels (Brand et al. 2010; Jonasson et al. 2010). Due to the wide geographical distribution of cyanobacteria and the possible implications of BMAA neurotoxic properties in public health, the study of the production of BMAA by cyanobacteria in estuarine ecosystems was needed. In this work, both free and protein-bound BMAA were analyzed in cultures representing the taxonomic diversity of Portuguese estuaries. BMAA was identified using an HPCL-FD method; nevertheless like BMAA has no chromophore, it needs to be derivatized in order to be detectable by fluorescence detection. We have used the highly reactive 6-aminoquinolyl-*N*-hydrosuccinimidyl carbamate (AQC) that reacts both with primary and secondary amines and yields fluorescent derivates. We have employed, a rapid and efficient HPLC-FD method using methanol elution instead of acetonitrile (Banack et al. 2007). Trichloroacetic acid (TCA), Methanol/Acetone and hydrochloric acid (HCl) have been evaluated to extract BMAA from cyanobacterial samples (Banack et al. 2007; Eriksson et al. 2009). Samples were collected and made unialgal by the standard methods (Lopes et al. 2010). Eighteen different strains were analyzed for the presence of BMAA, in what, as far as we know, is the first report of BMAA presence in estuaries.

Materials and methods

Cyanobacteria isolation and culture

Eighteen cyanobacterial strains were isolated from Portuguese estuaries, Minho, Douro and Vouga, located in the northwest of the country (coordinates are given in Table 1). The samples were collected during the low tidal from

Table 1 Taxonomic assignment and origin of each cyanobacterial strain

LEGE code	Taxa	Habitat	Estuary	Coordinates	
	Chroococcales				
06068	*Cyanobium* sp.	Benthos	Douro	41°8 ′50.77″N	8°39′12.89″W
06079	*Synechocystis salina*	Benthos	Douro	41°8′12.20″N	8°39′54.65″W
06083	*Synechocystis* cf. *salina*	Plankton	Douro	41°8′48.17″N	8°39′38.79″W
07073	*Synechocystis* cf. *salina*	Plankton	Vouga	40°40′16.42″N	8°43′24.36″W
07074	*Synechococcus* sp.	Benthos	Douro	41°8′48.17″N	8°39′38.79″W
	Oscillatoriales				
06069	*Leptolyngbya* sp. 1	Benthos	Douro	41°8′50.77″N	8°38′2.13″W
06070	*Leptolyngbya* sp. 2	Benthos	Douro	41°8′50.77″N	8°38′2.13″W
06072	*Phormidium*cf.*animale*	Benthos	Vouga	40°40′16.42″N	8°43′24.36″W
06078	*Phormidium* cf. *chalybeum*	Benthos	Douro	41°8′12.20″N	8°39′54.65″W
07075	*Leptolyngbya* sp. 2	Benthos	Douro	41°8′50.45″N	8°38′2.13″W
07076	*Microcoleus vaginatus*	Benthos	Minho	41°54′5.00″N	8°48′51.88″W
07080	*Leptolyngbya* sp. 1	Benthos	Minho	41°52′2.50″N	8°51′35.90″W
07084	*Leptolyngbya* sp. 1	Benthos	Minho	41°52′16.76″N	8°50′39.66″W
07085	*Leptolyngbya* aff. *bijugata*	Benthos	Douro	41°8′50.77″N	8°39′12.89″W
07091	*Leptolyngbya* sp. 1	Benthos	Vouga	40°40′16.42″N	8°43′24.36″W
07092	*Microcoleus chthonoplastes*	Benthos	Vouga	40°40′16.42″N	8°43′24.36″W
	Nostocales				
06071	*Nodularia* sp.	Benthos	Vouga	40°38′32.87″N	8°39′47.85″W
06077	*Nostoc* sp.	Plankton	Minho	41°52′40.13″N	8°50′6.33″W

benthos environments and water samples. Isolation and culture were performed in Z8 liquid medium (Kotai 1972) supplemented with NaCl (10–35 mg mL^{-1}). Isolation procedure was done with use of the micromanipulation technique of single cells using both liquid and solid medium (Ripka 1988). Cultures were grown under laboratory conditions at 25°C, light intensity of 20.8–27.4 × 10^{-6} E m^{-2} s^{-1} and a light/dark cycle of 14/10 h. Cultures examined were uni-cyanobacterial and non-axenic. Non-axenic and uni-cyanobacterial cultures were posteriorly lyophilized. The taxonomic characterization was performed as described before by Lopes et al. (2010).

BMAA extraction

Usually, BMAA extractions allow the distinction between two forms of BMAA, protein-bound and free, as the BMAA pool that is, or is not, bound to the protein fraction, respectively. In this work, two different sample extraction procedures were employed for free BMAA, one using trichloroacetic acid (TCA) (Banack et al. 2007), the other using Methanol/Acetone (Eriksson et al. 2009). The extraction of the protein-bound BMAA was achieved by hydrochloric acid (HCl) digestion (Banack et al. 2007).

TCA extraction

For each cyanobacteria sample, c.a. 17 mg was hand macerated with liquid nitrogen, weighed again and re-macerated in 0.1 M TCA (in a proportional part of 1 mL of TCA for each 400 µg of cyanobacteria sample), vortexed and extracted during 12 h at 4°C (Banack et al. 2007). After that, the sample was centrifuged at 13,800*g* for 5 min (Centrifuge 5415R, Eppendorf) and the supernatant decanted and preserved. The pellet was re-macerated with 50 µL 0.1 M TCA, vortexed, and re-extracted for 1 h at room temperature. Samples were centrifuged at 13,800*g* for 5 min. The TCA supernatants were pooled and concentrated in a speed-vacuum (Concentrator Plus Complete System). Final volume was measured for posterior calculations. The sample was derivatized with AQC (Cohen 2001).

Methanol/Acetone extraction

Twenty milligrams of cyanobacteria sample was dissolved in 450 µl of 70% methanol (70/30 methanol:water) and sonicated (Bandelin, Sonorex-RK100H). Ice-cold acetone (−20°C) was added with 4× the volume of the lyzed cell suspension and vortexed briefly (Eriksson et al. 2009). Immediately, the sample was conserved at −20°C for 3 h, for allowing it to precipitate. After that, the samples were centrifuged at 12,000*g* for 10 min (Centrifuge 5415R, Eppendorf). The supernatant was separated and transferred to a new tube (it is the free BMAA). Both fractions were dried using a speed-vacuum (Concentrator Plus Complete System). The dried supernatant was re-suspended in 20 mM HCl (100 µL) and derivatized with AQC (Cohen 2001) and the residual pellet gotten was saved for the analysis of protein-bound BMAA.

HCl extraction

Twenty milligrams dry weight of each cyanobacteria sample was digested in a vacuum atmosphere, by acid hydrolysis with 6 M HCl (320 µL) at 150°C for 2 h (Banack et al. 2007). In the residual pellet is found the total BMAA. The residual pellet was dried using a speed vacuum (Concentrator Plus Complete System) and re-suspended in 20 mM HCl (100 µL), filtered (0.22 µm Ultrafree-MC, Millipore), and centrifuged at 13,800*g* for 3 min (Centrifuge 5415R, Eppendorf). The sample was diluted 1:10 using 20 mM HCl before derivatization with AQC. On the other hand, the residual pellet resulting from the Methanol/Acetone extraction (Eriksson et al. 2009) was likewise digested.

Derivatization reaction

Derivatization was carried out using AQC, as described by Cohen (2001). To 10 µL of the sample, were added 70 µL of 0.5 M borate and 20 µL of AQC. After AQC addition, the sample was allowed to react for 1 min, at room temperature. The sample was transferred to an autosampler vial and heat at 55°C for 10 min, in a test tube SHT 1D Stuart Scientific.

BMAA quantification

Derivatized samples were analyzed by injecting 5 µL of sample in a HPLC-FD system. BMAA–AQC was separated using a Waters Nova-Pak C18 column, 3.9 × 300 mm, coupled with a Waters guard column (WAT046905), on a Merck Hitachi L-7100 HPLC pump and a Merck Hitachi L-7200 autosampler. The BMAA concentration was quantified by detection of the fluorescent tag in a Merck Hitachi L-7480 multi λ-fluorescence detector, with excitation at 250 nm and emission at 395 nm. Data were analyzed with the Merck Hitachi Model D-7000 chromatography data station software.

For BMAA measurement, a gradient method with 140 mM sodium acetate, 5.6 mM triethylamine, (Eluent A), and 65% methanol in water (Eluent B), was employed. The separation was performed at 37°C, with a flow rate of 1 mL min^{-1}. For free BMAA measurement eluent A had pH 5.2 and the separation was performed for 30 min (0.0 min = 75% A; 17.0 min = 63% A; 18.8 min = 100%

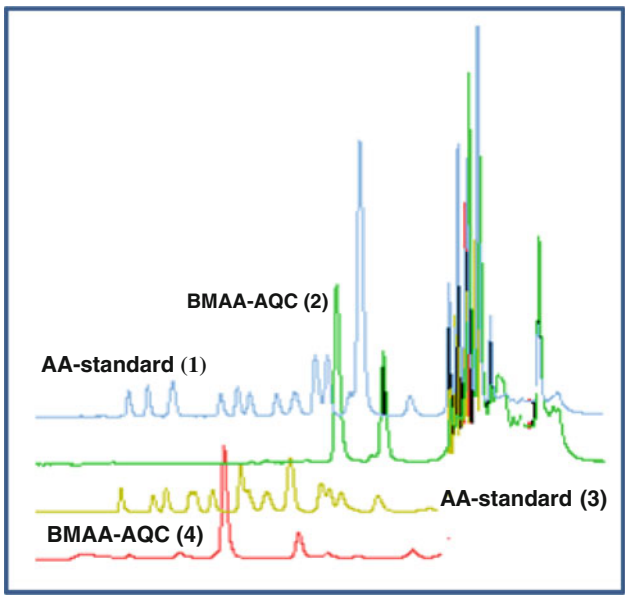

Fig. 1 HPLC-FD chromatograms using free (*3* and *4*) and protein-bound (*1* and *2*) BMAA methods. Although the two different HPLC-FD analyses use different gradients, they were united using a sodium acetate buffer and fluorescent detection. We can observe that there is not a coelution of the BMAA-AQC standard with amino acid standard in any of the two methods employed. The BMAA concentration employed for free BMAA was 0.85 nM, whereas for protein-bound BMAA it was 8.5 nM. On other hand, for AA standard solution, we used 25 and 100 pM/μL in each case

A; 23.5 min = 100% A; 25 min = 75% A; 30.0 min = 75% A) (Banack et al. 2007), whereas for the total BMAA measurement eluent A had pH 5.7 and the separation was performed for 50 min (0.0 min = 100% A; 0.5 min = 98% A; 18.0 min = 93% A; 19.0 min = 90% A; 29.5 min = 83% A; 33.0 min = 67% A; 34.0 min = 0% A; 37 min 0% A; 38 min = 100%; 50.0 min = 100%) (Cohen 2001). The pH was adjusted with a 10% phosphoric acid solution.

Derivatized samples were run in parallel with water blanks, derivatized acid blanks, and derivatized BMAA standards (Sigma Aldrich, L-BMAA hydrochloride) to ensure the efficiency of the BMAA extraction. Derivatized amino acid calibration stock solution, and derivatized amino acid calibration stock solution spiked with BMAA were run to confirm the separation and correct identification of the BMAA peak (Fig. 1). LOD (Limit of detection) and LOQ (Limit of quantification) were 0.13 pM and 0.42 pM for free BMAA and 0.06 and 0.19 pM for the total BMAA.

Results and discussion

BMAA extraction

The cyanobacteria collected and isolated from the Portuguese estuaries of Minho, Douro and Vouga Rivers were identified and could be grouped in three different orders: Chroococcales, Oscillatoriales and Nostocales (Table 1).

Three different extraction procedures were employed and compared in this study. The three extraction procedures enabled the detection of BMAA, although the values of BMAA obtained with each extraction procedure varied. Figure 2 shows that considerable difference in BMAA content was found between the extracts. Free BMAA, defined as the fraction of BMAA released after Methanol/Acetone or TCA extraction and assumed as not bound to the protein pool, was higher after extraction with TCA (Fig. 2b). Protein-bound, or the total, BMAA was the fraction released after the extraction with hot HCl, usually assumed to digest the entire protein content of the sample. This extraction was applied sequentially after extraction with Methanol/Acetone (Fig. 2a) and applied to the cyanobacteria lyophilized samples (Fig. 2c).

Three extraction procedures used in this work have been proposed for the routine determination of BMAA (Banack et al. 2007; Eriksson et al. 2009) with good results in terms of BMAA detection. However, to our knowledge, these procedures had never been applied to the same samples. About the great differences detected in BMAA values using the above extraction procedures; we tried to infer that step could be the responsible of the detected differences. We know that the first step in the isolation of a protein is its liberation from the cells. For free BMAA, the method employed in both cases was the mechanical rupture with liquid nitrogen and sonication (Eriksson et al. 2009) or maceration (Banack et al. 2007). In the first case, Methanol/Acetone was used for extract free BMAA and precipitate the protein, in the second one was used TCA. After that, both procedures use cold temperatures (−20°C for Eriksson's method and 4°C for Banack's method) for facilitate the protein precipitation and centrifugation for its separation. On other hand, with respect protein-bound BMAA levels, great differences also were detected, but with Banack et al.'s (2007) method measuring was for the total BMAA (cyanobacteria lyophilized samples) and with Eriksson et al.'s (2009) method was just for protein-bound BMAA (analyzed in the residual pellet), in both cases the methodology was the same, hydrolysis acid with 6 M HCl. Finally, with the two extraction procedures all the extracted samples (supernatants and residual pellets) were concentrated and evaporated in a speed vacuum and later derivatized. Methanol/Acetone and TCA are used indistinctly for the denaturalization of proteins. So that, we could wait that in the analyzed samples the free BMAA values were very similar. Nevertheless is not the case. After the previous analysis of both extraction procedures, we can observe that there is a great similitude in both processes. The just one difference is the employed reagents. So, we think that the principal difference could be the final pH

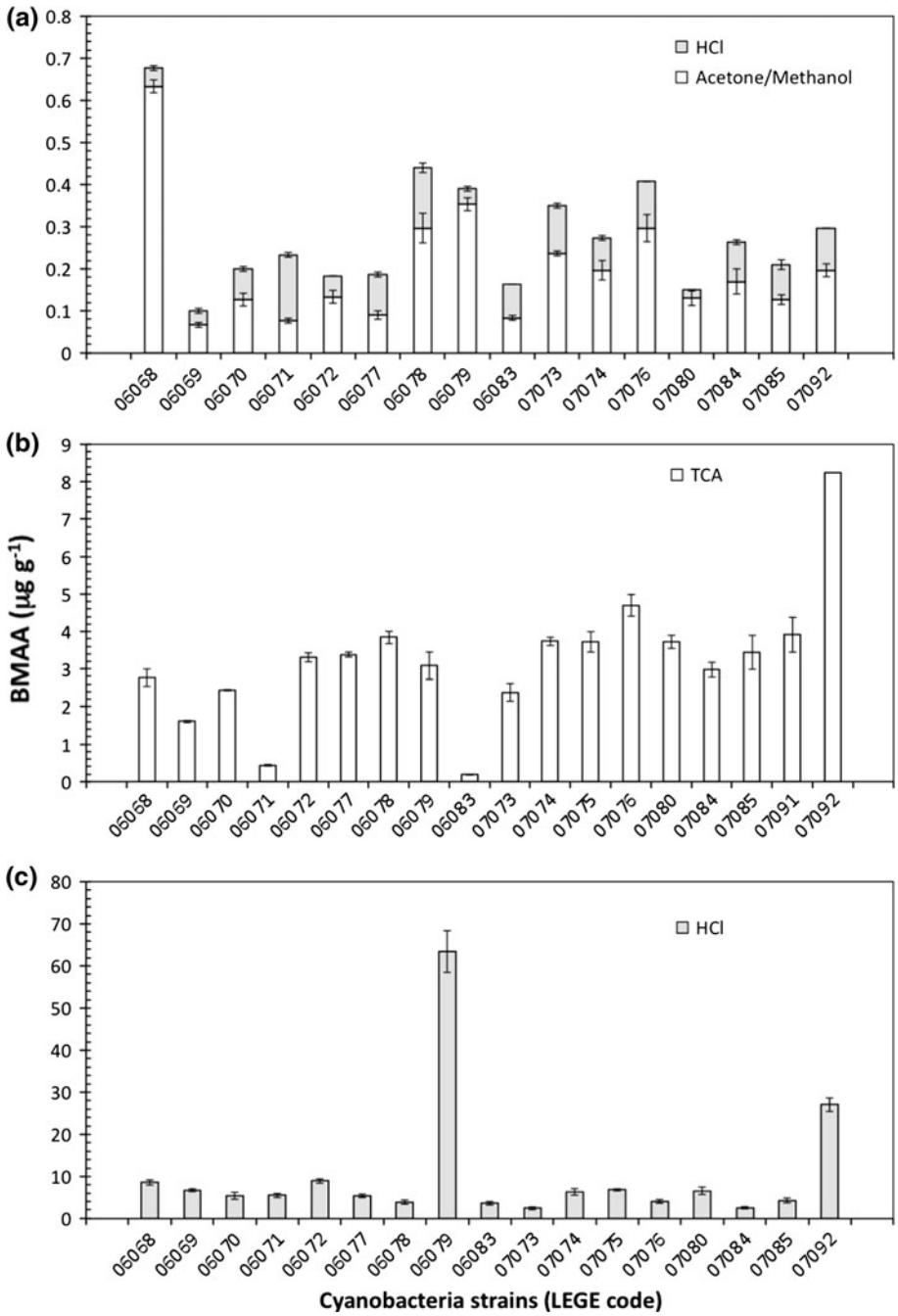

Fig. 2 BMAA measured in estuarine Portuguese cyanobacteria following different extraction procedures: (**a**) Methanol/Acetone extraction, followed by HCl extraction, (**b**) TCA extraction, (**c**) HCl extraction. *Values* are average of three replicates and *bars* are standard deviation

gotten after that Cohen and Michaud (1993) mentioned that studies on derivatization conditions demonstrate excellent derivative yield over the pH range 8.2–10.0. So, an improper or inadequate sample buffering during the AQC derivatization could produce that BMAA be partially detected. It might be the case of the differences detected with both extraction procedures. The results gotten show that the extraction of BMAA from a cyanobacteria sample is greatly depending on the reagent used. In our case, we consider that TCA extraction is the better method to evaluate the free BMAA containment. Furthermore, other authors had already concluded, by comparing different methods of analysis that the BMAA values found in cyanobacteria samples may vary according the analytical method (Banack et al. 2010). Standardization of BMAA analysis in cyanobacteria will be essential for environmental risk analysis of this putative neurotoxin. Within this context, the first step to be standardized must be the extraction procedure. The influence of the extraction reagent has been shown in this work, but other questions should be addressed when comparing different results obtained by different groups. The physical characteristics

of the samples might also play a role in the extraction procedure. For instance, Eriksson et al. (2009) employed the Methanol/Acetone plus HCl extraction using freshly collected cyanobacteria, from culture medium, whereas in this work the samples had been previously lyophilized for all the extracted procedures employed.

BMAA quantification in estuarine cyanobacteria

During the change of the mobile phase from acetonitrile to methanol, the great problem was the coelution of BMAA with some amino acids. After different assays (data not showed), we could separate BMAA and avoid the coelution. We have not identified the amino acids present in the standard, because the only objective was to separate BMAA from them (Fig. 1). On other hand, in this work, the 18 analyzed strains encompassed eight genera included in three orders (Table 1). Analysis of BMAA content by taxonomic group did not reveal an overall tendency for any genera to be a higher BMAA producer (Fig. 3). The quantification of BMAA in different strains of cyanobacteria has revealed so far, great diversity of BMAA content, in samples collected from marine, brackish water and freshwater (Cox et al. 2005; Esterhuizen and Downing 2008; Metcalf et al. 2008). This work was no exception and the values obtained varied from 0.04 μg g^{-1} for *Cyanobium* sp. sequential extraction with Methanol/Acetone plus HCl to 63 μg g^{-1} for *Synechocystis salina* extraction with HCl (Fig. 2).

Analysis of BMAA content by geographical region did not reveal a tendency for any estuary to be a higher BMAA producer either (Fig. 3). In Portugal, as in many other countries, estuaries encompass areas of high population density. Assertion of BMAA production in estuaries is, therefore, as more relevant as the number of people that can be impacted. So far, the health risk to humans from the direct exposure to BMAA in water is unknown. BMAA in estuarine waters has not been reported, and given the hydrophilic character of the amino acid it is likely that the current limits of detection of chromatographic techniques (the most usually employed BMAA analysis techniques) are not low enough to detect its occurrence. However, the fate of BMAA in the aquatic environment might also pass through biomagnification. For marine waters, two works have recently reported biomagnification across trophic chains of the North Atlantic (Jonasson et al. 2010; Brand et al. 2010). As pointed by other authors, the potential for biomagnification through the food chain remains as a possible mechanism for human exposure to large amounts of BMAA, which might contribute to the development of neurodegenerative diseases (Karamyan and Speth 2008). For these reasons, the fate and transport of BMAA produced by estuarine cyanobacteria deserves more attention.

Conclusions

BMAA production from naturally occurring populations of cyanobacteria has been shown, for a number of strains that encompasses great diversity within cyanobacteria. Three different extraction techniques have been compared for this effect, Trichloroacetic acid (TCA), Methanol/Acetone and hydrochloric acid. HPLC-FD using methanol instead of acetonitrile let BMAA identification. In Portuguese estuaries, the free and protein-bound BMAA content of cyanobacteria samples varied with the location and the taxa.

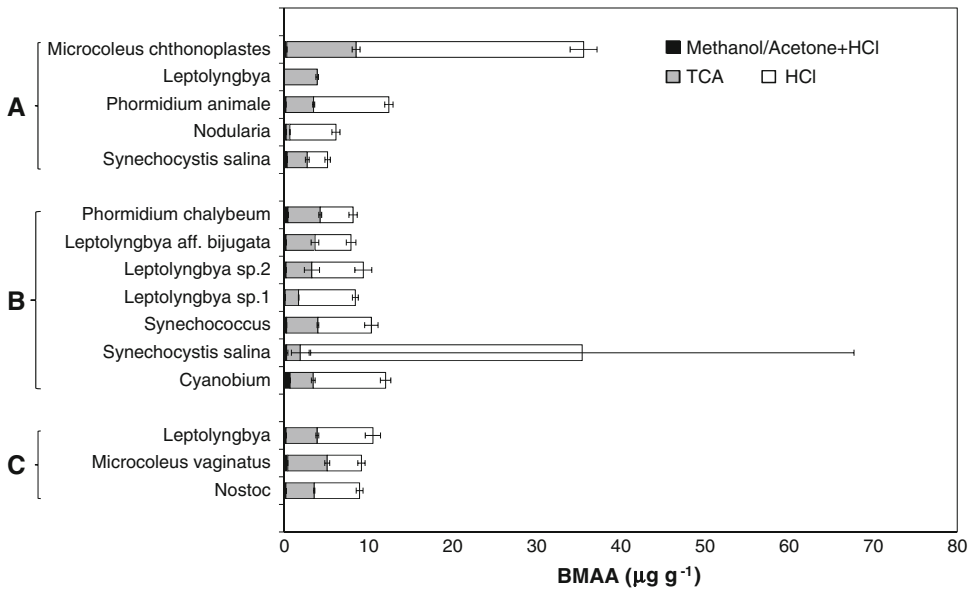

Fig. 3 BMAA content measured in cyanobacteria isolated grouped by geographic location. Vouga (**a**), Douro (**b**) and Minho (**c**) estuaries. Methanol/Acetone + HCl consider free and protein-bound BMAA

BMAA potential biomagnification in edible aquatic animals might pose a human health hazard and further assessment of estuaries, in terms of BMAA occurrence, is needed.

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