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Communication

No β-N-Methylamino-L-alanine (BMAA) Was Detected in Stranded Cetaceans from Galicia (North-West Spain)

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Abstract: The neurotoxin β-N-methylamino-L-alanine (BMAA), a non-proteinogenic amino acid produced by several species of both prokaryotic (cyanobacteria) and eukaryotic (diatoms) microorganisms, has been proposed to be associated with the development of neurodegenerative diseases. At first, BMAA appeared to be ubiquitously present worldwide in various organisms, from aquatic and terrestrial food webs. However, recent studies, using detection methods based on mass spectrometry, instead of fluorescence detection, suggest that the trophic transfer of BMAA is debatable. This study evaluated BMAA in 22 cetaceans of three different species (*Phocoena phocoena*, $n = 8$, *Delphinus delphis*, $n = 8$, and *Tursiops truncatus*, $n = 6$), found stranded in North-West Spain. BMAA analysis of the liver, kidney, or muscle tissues via sensitive liquid chromatography with tandem mass spectrometry did not reveal the presence of this compound or its isomers. The absence recorded in this study highlights the need to better understand the trophic transfer of BMAA and its anatomical distribution in marine mammals.

Keywords: marine mammals; phycotoxins; harmful algae blooms; bioaccumulation; marine food webs; Alzheimer disease

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1. Introduction

The non-proteinogenic neurotoxin β-N-methylamino-L-alanine (BMAA) has been proposed to act as an environmental factor, inducing the development of several neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease, and Parkinson's disease [1–4]. However, the hypothesis of a causative association between dietary exposure to BMAA and a neurodegenerative pathological condition remains controversial [5–8]. Cox et al. (2005) [9] detected BMAA in 95% of all cyanobacterial genera tested and concluded that BMAA was universally produced by cyanobacteria species from aquatic and terrestrial habitats, in both symbiotic and free-living forms. However, this conclusion was questioned, due to the non-specificity of the analytical method used. The positive detection of BMAA, with liquid chromatography (LC) or gas chromatography (GC), associated with ultraviolet, fluorescence spectroscopy, or single mass spectrometry (MS), is just based on the retention time and signal of the parent ion [10–12]. All these analytical methods might give false-positive results, considering that BMAA might co-elute with its natural isomers (i.e., DAB, 2,4-diaminobutyric acid; BAMA, β-amino-N-methyl-alanine; AEG, N-2(aminoethyl)glycine; DABA, 2,3-diaminobutyric acid; 3,4-diaminobutyric

acid; 3-amino-2-(aminomethyl)-propanoic acid; and 2,3-diamino-2-methylpropanoic acid) or other interfering compounds [13]. Currently, it is accepted that only the use of liquid chromatography with tandem mass spectrometry detection (LC-MS/MS), with or without previous derivatization, ensures a reliable BMAA identification, based on the retention time, mass-to-charge ratio (m/z) of the precursor ion, and product fragmentions after collision-induced dissociation, and the ratio between the intensities of respective ions transitions in multiple reaction monitoring (MRM) spectrum [10,13]. Moreover, recent studies, using LC-MS/MS, have shown that most cyanobacteria produce only trace levels of BMAA [14,15].

In addition to cyanobacteria, several diatoms species produce BMAA and its structural isomers [16–18]. Several of these potential BMAA-producers are present in our study area, the coast of Galicia in North-West (NW) Spain (Figure 1), i.e., the diatoms *Chaetoceros* spp., *Navicula* spp., *Skeletonema* spp., and *Thalassiosira* spp. [19–23]. and cyanobacteria from the genera *Anabaena*, *Myxosarcina*, *Lyngbya*, *Phormidium*, *Symploca*, *Nodularia*, *Nostoc*, *Calothrix*, and *Microcystis* [20]. Moreover, the predominant wind and oceanographic conditions of NW Spain enhance phytoplankton production, and diatoms and dinoflagellates blooms are recurrently reported [20].

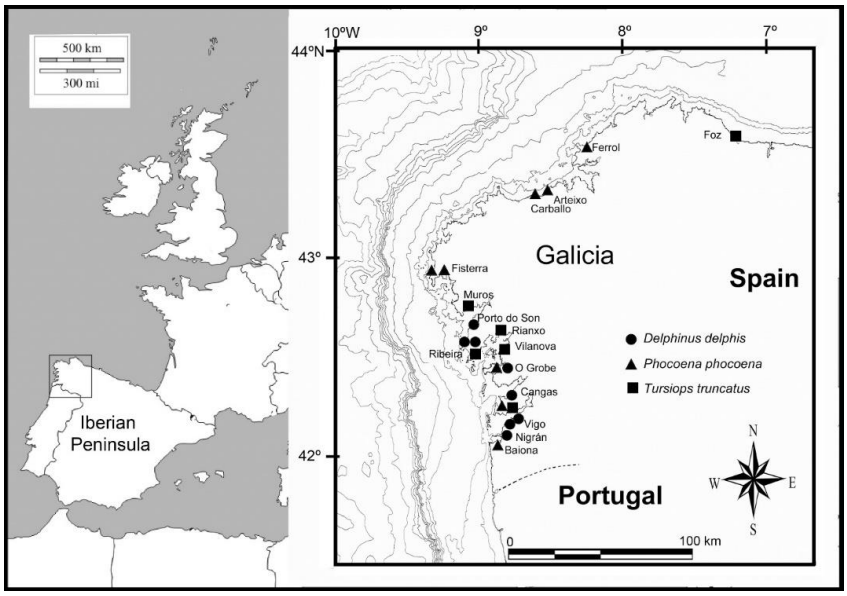

Figure 1. Location of stranded marine mammals collected along Galician coast that were used in the present study.

The presence of BMAA has been described in several organisms along the aquatic and terrestrial food chains, including zooplankton [24–26], crustaceans [27,28], bivalves [25,29–31], fish [25,32], and terrestrial plants and animals [33–36]. However, a review by Lance et al. (2018) [37] observed no clear indication of the BMAA trophic transfer. If BMAA is bioaccumulated through the marine food web, it is plausible that the highest concentration of BMAA would be present in apex predators [38–40]. Massive strands of marine mammals, due to harmful algal events, have been reported worldwide, and evidences of their recurrent exposure to these toxins was also suggested [41–44]. Cetaceans are important key species for marine ecosystems, being excellent indicators of environmental changes. Indeed, recent studies performed in the West Atlantic confirmed that common and bottlenose dolphins are susceptible to BMAA accumulation and damage [8,45].

Galicia, located in NW Spain (Figure 1), holds important populations of marine mammals, which reckon 23 species [46,47]. Among them, the common dolphin (*Delphinus delphis*), bottlenose dolphin(*Tursiops truncatus*), and harbour porpoise (*Phocoena phocoena*) are the most frequently found washed ashore [48–50]. The harbour porpoise in the area has been identified as a new ecotype and proposed as a new subspecies [51].

Therefore, to explore the potential BMAA trophic transfer, and its potential association with the stranding of cetaceans, we analysed the BMAA, DAB, and AEG levels in three different tissues (liver, kidney, and muscle) of 22 individuals from three cetacean species, namely the common dolphin, bottlenose dolphin, and harbour porpoise. All individuals were found stranded between 2011 and 2017.

2. Materials and Methods

A stranding network was established in Galicia, in 1990, and carried out by NGO Coordinadora para o Estudo dos MamíferosMariños (CEMMA), to locate beached cetaceans, pinnipeds, and sea turtles, ensuring the biological samples collection and rehabilitation actions for live animals. Basic data recorded includes species, biometrics, gender identification, body condition, external examination for each animal, and signs of bycatch. Any other relevant details were also recorded (Table 1). Necropsies were carried out on fresh and moderately decomposed animals. Both external studies and necropsies followed standardized protocols [52–54]. Figure 1 displays the location where the animals, used in this study, were found.

Table 1. Individual data of animals analysed for BMAA, DAB, and AEG. For each animal, samples of liver, kidney, and muscle were collected and analysed. Degradation state: 1 = found alive, dying immediately afterwards, 2 = freshly dead, 3 = moderate decomposition, 4 = advanced decomposition, 5 = skeletal remains. F = female, M = male.

Sample ID	Species	Data of Collection (yyyy/mm/dd)	Location (Locality and Coordinates)	Size (cm) *	Female (F)/Male (M)	Degradation State	Observations
DDE194	*D. delphis*	2017/07/16	Porto do Son (42.68131, −9.03000)	194	F	3	No signs of bycatch
DDE195	*D. delphis*	2017/07/16	Ribeira (42.56142222, −8.987644444)	195	M	3	No signs of bycatch
DDE189	*D. delphis*	2017/07/31	Cangas (42.24911, −8.79091)	189	F	3	Signs of bycatch
DDE182	*D. delphis*	2017/07/31	Nigrán (42.14245, −8.83796)	182	M	3	Three broken ribs and subepidermic hematoma
DDE173	*D. delphis*	2018/06/09	Ribeira (42.57156, −9.07536)	173	M	1	No signs of bycatch
DDE156	*D. delphis*	2018/11/07	Vigo (42.19025, −8.80904)	156	M	2	-
DDE124	*D. delphis*	2018/11/25	O Grove (42.45576667, −8.921633333)	124	F	3	Signs of bycatch
DDE172	*D. delphis*	2018/12/17	Vigo (42.22298056, −8.772766667)	172	F	3	Signs of bycatch
PPH153	*P. phocoena*	2009/11/25	Fisterra (42.941181, −9.231806)	153	F	3	Good aspect
PPH127	*P. phocoena*	2011/02/07	Fisterra (42.908431, −9.258883)	127	M	3	Signs of bycatch
PPH104	*P. phocoena*	2011/05/27	Baiona (43.056527, −9.295471)	104	F	2	Signs of bycatch
PPH137	*P. phocoena*	2012/12/02	Arteixo (43.316153, −8.534233)	137	F	3	Signs gulls and shark bites
PPH142	*P. phocoena*	2013/10/15	O Grove (43.463064, −8.332825)	142	F	3	-
PPH159	*P. phocoena*	2014/02/28	Ferrol (43.540556, −8.298353)	159	M	3	Signs of bycatch
PPH131	*P. phocoena*	2015/01/16	Cangas (42.261026, −8.849869)	131	M	3	Signs of bycatch
PPH160	*P. phocoena*	2017/02/20	Carballo (43.456950, −8.673272)	160	M	3	Signs of bycatch
TTR258	*T. truncatus*	2015/10/30	Ribeira (42.523304, −9.014026)	258	M	3	Stomach with food and Anisakis, ulcers
TTR314	*T. struncatus*	2015/12/30	Vilanova de Arousa (42.570726, −8.830053)	314	F	2	Empty stomach
TTR286	*T. truncatus*	2016/09/02	Cangas (42.295522, −8.820978	286	F	3	Pregnant
TTR150.5	*T. truncatus*	2016/09/11	Rianxo (42.64833056, −8.8258)	150.5	M	3	Signs of aggressions (possible infanticide)
TTR275	*T. truncatus*	2017/06/19	Foz (43.5665472, −7.2546361)	275	F	2	Skinny
TTR138	*T. truncatus*	2018/11/30	Muros (42.7747527, −9.05476)	−138	M	4	Signs of bycatch

* The sign (-) indicates the animal is not entire, and the size is at least the indicated measure.

Tissue samples of liver, kidney, and muscle were frozen at −20 °C for preservation. Before analyses, the samples were freeze-dried (Freeze Dry System Labconco, Coolvacuum Technologies, Barcelona, Spain). The BMAA (total soluble and precipitated bound) was extracted, based on Murch et al. (2004) [55], with minor alterations, suggested byMasseret et al. (2013) [56] and Lage et al. (2016) [57], as previously described [29,57,58]. The tissue samples (2 mg dry weight each) were extracted in triplicate. After extraction, freeze-dried BMAA total soluble and precipitated bound samples were reconstituted with 20 mM HCl solution and dilutions were performed (if required), to obtain an optimum ratio of protein-to-derivatization agent ratio [57]. Subsequently, the samples were derivatized with AccQ-Tag, using a WAT052880 AccQ-Tag kit (Waters, Milford, MA, USA) before analysis.

Samples of each tissue type (liver, kidney, and muscle) and BMAA form (i.e., total soluble and precipitated bound)were used for the analysis of limits of quantification; LOQ was defined as S/N $\geq$ 10 [59]. Samples of liver BMAA precipitated bound form were used for the evaluation of the matrix effect and extraction method recovery at the BMAA concentrations of 5 and 10 ng mL^{-1} (n = 4).

LC-MS/MS analysis of derivatized BMAA, and its isomers AEG and DAB, were performed in an Acquity UPLC system, coupled witha Xevo-TQ-MS system (Waters, Milford, MA, USA), as previously described [30].The LC separation was performed on an AccQ-Tag Ultra C18 column (100 × 2.1 mm, 1.7 µm particle size, Waters, Milford, MA, USA). Ionization was performed in positive ion mode, and the mass analyser was run in the selected reaction monitoring (SRM) scanning mode, using the following transitions to distinguish BMAA from its isomers, AEG and DAB: common to all three analytes, 459.1 > 119.1 (CE 30.0); DAB diagnostic fragment, 459.1 > 188.1(CE 38.0); BMAA diagnostic fragment, 459.1 > 258.1 (CE 30.0); and AEG diagnostic fragment, 459.1 > 214.1 (CE 30.0). To ensure the accurate identification of BMAA, the parameters retention time and fragmentation ratio of the fragments, 119.1/258.1, were considered. All settings were optimized for the detection of BMAA, as follows: spray voltage, 5500 V; source temperature, 450 °C; decluttering potential, 50; focusing potential, 350; and entrance potential, 6.MassLynx V4.1 software (Waters, Milford, MA, USA) was used to analyse the acquired data.

3. Results and Discussion

No measurable levels of BMAA and its structural isomers (AEG and DAB) were detected, in either the total soluble or precipitated bound forms of the three tissues (liver, kidney, and muscle) of the 22 specimens studied. To the best of our knowledge, this is the first study analysing the liver, kidney, and muscle tissue samples of cetaceans for the presence of BMAA and its structural isomers. Previously, Davis et al. (2019) reported total BMAA concentrations, ranging from 20 to 748 μg^{-1}, in the brains of 13 dolphins, found stranded in Florida and Massachusetts, USA. Furthermore, dolphins with detectable levels of BMAA presented injuries in the cerebral cortex and increased β-amyloid plaques [8]. Unfortunately, the discrepancy in dolphins' tissues, analysed by Davis et al. (2019) (i.e., brain) and the current study (i.e., liver, kidney, and muscle), does not allow a direct comparison.

Although the brains of the animals were not available for the present study, considering the lack of data on BMAA accumulation in marine mammals, we judged that the analyses of other tissues may still be of relevance. In fish species, the four tissues (brain, liver, kidney, and muscle) have been previously analysed, thus providing an estimation of the BMAA anatomical distribution [25,60]. In several fish species, collected in the Baltic Sea and in Lake Finjasjön (Sweden), the highest BMAA levels were found in the fish brains [25,60]. The total BMAA concentrations in the fish brains were up to 0.99 and 0.028 µg g^{-1} DW, while in the fish muscle were up to 0.059 and 0.006 µg g^{-1} DW in the Baltic Sea and Lake Finjasjön, respectively [25,60]. Moreover, from the total of 136 fish individuals analysed by Lage et al. (2015), only 22 individuals (16%) contained quantifiable BMAA in their muscle, while BMAA was quantified in the brains of 40 individuals (29%) [60]. No BMAA was detected in the kidney and liver of fish collected in the Baltic Sea and Lake Finjasjön [25,60].

In other studies of fish collected in the Baltic Sea, the Eastern North Atlantic, and the Mediterranean Sea, no BMAA was detected in the fish muscle [16,26,28,61].

Tissue composition may play an important role in BMAA accumulation. BMAA is misincorporated, instead of L-serine during protein synthesis. Moreover, BMAA anatomical distribution in adult mice showed a distribution pattern analogous to protein-forming amino acids [62,63]. Another study in neonatal rats reported a higher uptake and retention of BMAA in tissues, with high rates of protein synthesis and cell turnover, suggesting that BMAA may be incorporated or associated with newly synthesized proteins [64]. However, BMAA was cleared out of the body over time. Thus, the non-detection of BMAA in the muscle, liver, and kidney of cetaceans found stranded in Galicia might be due to the higher turn-over rate of these tissues, leading to the degradation and release of BMAA, especially if the cetaceans were starved for a long period of time.

Although brain samples were not analysed, the muscle of several fish species caught in Lake Taihu (China) had total BMAA concentrations higher than the muscle of fish caught in the Baltic Sea, Eastern North Atlantic, and Mediterranean Sea, with concentrations ranging from 0.07 and 35.91 μg g^{-1} DW [65]. Moreover, sharks, apex predators caught in South Florida (USA), had total BMAA concentrations between 19.2 and 33.15 μg g^{-1} FW in the fins and muscle [32,66]. Furthermore, dietary supplements containing shark cartilage, from various species and origins (not reported), had total BMAA concentrations between 74.8 and 352.2 μg g^{-1} DW [67]. BMAA contents in organisms may vary, depending on the methodological differences between studies, inter-specific variations in their trophic status, geographical and seasonal parameters, and ecological responses of its producers [37]. Thus, as previously documented for fish [16,25,26,28,32,60,61], dolphins of certain geographical areas might also contain higher concentrations of BMAA than others. Accordingly, Davis et al. (2019) reported three-fold higher concentrations of total BMAA in dolphins stranded in Florida than in dolphins from Massachusetts [45].

Methodological differences between studies are often responsible for the variability of data reported on BMAA concentration in biota, especially when dealing with complex tissues, which may induce matrix interferences [10,37,68]. A previously published and in-house validated LC-MS/MS method [56,57,69], which had minor differences from the method used by Davis et al. (2019,2021), was used in the present study [8,45]. The LOQ on BMAA spiked tissue samples was 0.5 ng mL^{-1} (corresponding to 0.11 μg g^{-1}), and the matrix effect in the liver tissue precipitated bound BMAA samples was 64.04 ± 3.77 and 54.22 ±6.14% in the 5 and 10 ng mL^{-1} spiked concentrations, respectively (Figure 2). The BMAA recovery rates in the 5 and 10 ng mL^{-1} spiked BMAA liver samples were 96.62 ± 20.12 and 99.20 ± 17.44%, respectively. Davis et al. (2019, 2021) reported a LOQ of 7.0 ng mL^{-1} and an average % recovery of BMAA of 98.3% [8,45]. Moreover, matrix spiked recovery (%) of several metabolites has been previously shown to be similar among various cetaceans tissues, i.e., blubber, muscle, liver, kidney, stomach, melon, and gonad [70]. Therefore, if the tissues analysed in the present study had BMAA concentrations comparable with the concentrations reported for the brains of cetaceans stranded in the USA, they would have been quantified. Unfortunately, as we did not analyse brain tissues or cerebral spinal fluid, we cannot rule out higher toxin levels in these tissues.

The absence of BMAA in our samples is unexpected. In Galicia, the phytoplankton successions, during the upwelling system dynamics, are characterized by the dominance of diatoms from late-winter to summer [19,20]. Among these diatoms, several potential BMAA-producers are accounted reaching high densities [19–23]. The estuaries ("rías") receive nutrients and phytoplankton from rivers, and high abundances of cyanobacteria, including potential BMAA-producers, are frequent in moderately stratified waters [71–75]. Unfortunately, BMAA levels for these bloom periods are unknown. Regulated toxins, such as amnesic toxins (domoic acid), paralytic shellfish toxins (saxitoxins), and the diarrhetic shellfish toxins (okadaic acid and derivatives), are regularly monitored, according to EU directives, to ensure human food safety [76]. However, BMAA lacks specific regulations and monitoring plans.

The BMAA absence, recorded in this study, highlights the need to better understand the trophic transfer of BMAA, depending on environment conditions and its anatomical distribution in marine mammals.

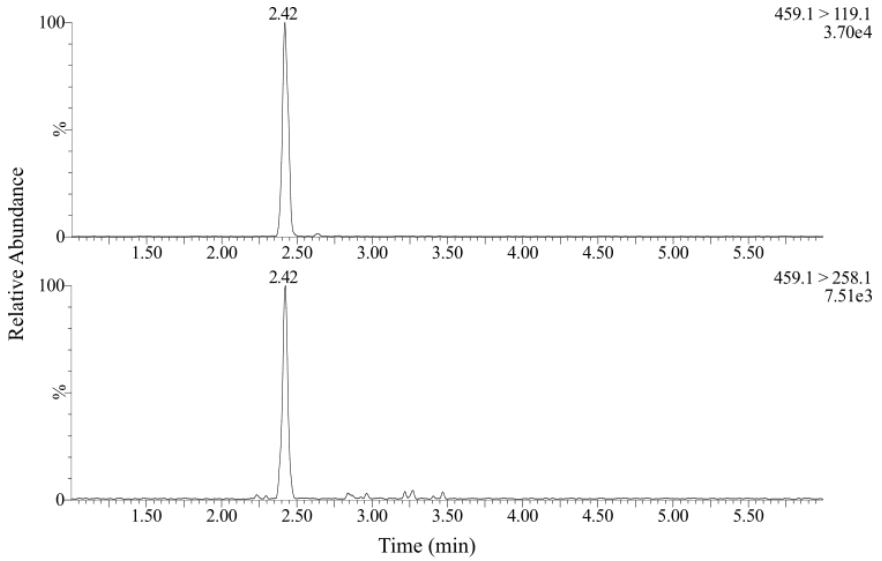

Figure 2. LC–MS/MS chromatograms of liver tissue precipitated bound sample extract spiked with 5 ng^{-1} mL of BMAA. The selected reaction monitoring (SRM) transitions 459.1 > 119.1 (common to BMAA and its isomers) and 459.1 > 258.1 (BMAA diagnostic fragment) are shown.

4. Conclusions

Neither the biotoxin BMAA nor their isomers were detected in several specimens of dolphins washed ashore in the Galicia coastline, Spain. Top predators, such as cetaceans, may reflect the overall BMAA incidence in the marine environment, which makes them suitable sentinel species for biotoxin exposure risk. The absence of BMAA in the liver, kidney, and muscle of the 22 individuals analysed might indicate a lower incidence of BMAA producers in this geographical area or lack of BMAA trophic transfer, but research on differential toxin accumulation in tissues and organisms needs further attention.

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Conflicts of Interest: The authors declare no conflict of interest.

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