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BMAA in shellfish from two Portuguese transitional water bodies suggests the marine dinoflagellate *Gymnodinium catenatum* as a potential BMAA source

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ABSTRACT

The neurotoxin β-N-methylamino-L-alanine (BMAA) and its putative role in multiple neurodegenerative diseases have been intensely studied since 2005 when the toxin was discovered to be produced by worldwide-distributed cyanobacterial species inhabiting terrestrial, marine, brackish, and freshwater ecosystems. Recently, BMAA production was also associated with one eukaryotic group, namely, diatoms, raising questions about its production by other phytoplanktonic groups. To test for BMAA bioavailability in ecosystems where abundant phytoplanktonic blooms regularly occur, samples of filter-feeding shellfish were collected in two Portuguese transitional water bodies. BMAA content in cockles (*Cerastoderma edule*) collected weekly between September and November 2009 from Ria de Aveiro and at least once a month from May to November from Ria Formosa, fluctuated from 0.079 ± 0.055 to 0.354 ± 0.066 μg/g DW and from below the limit of detection to 0.434 ± 0.110 μg/g DW, respectively. Simultaneously to BMAA occurrence in cockles, paralytic shellfish toxins were detected in shellfish as a result of *Gymnodinium catenatum* blooms indicating a possible link between this marine dinoflagellate and BMAA production. Moreover, considerable high BMAA levels, 0.457 ± 0.186 μg/g DW, were then determined in a laboratory grown culture of *G. catenatum*. This work reveals for the first time the presence of BMAA in shellfish from Atlantic transitional water bodies and consubstantiate evidences of *G. catenatum* as one of the main sources of BMAA in these ecosystems.

1. Introduction

β-N-methylamino-L-alanine (BMAA) is a neurotoxic non-protein amino acid with a proposed link to neurodegenerative diseases (Banack and Cox, 2003; Banack and Murch, 2009; Bradley and Mash, 2009; Cox et al., 2003; Hill, 1994). BMAA was first discovered in 1967 on the island of Guam (Vega and Bell, 1967), where it was originally isolated from the seeds of *Cycas circinalis*.

The BMAA source in Guam was later rectified, to the nitrogen-fixing symbiotic cyanobacterium *Nostoc* sp. (Cox et al., 2005). In 2005, Cox et al. reported that over 95% of all analyzed cyanobacterial strains from all five taxonomic sections produce BMAA (Cox et al., 2005). Subsequently, several studies of potential BMAA-producing species have confirmed the global extent of BMAA-producing cyanobacteria in diverse ecosystems (Craighead et al., 2009; Esterhuizen and Downing, 2008; Faassen et al., 2009; Johnson et al., 2008; Jonasson et al., 2010; Li et al., 2010; Metcalf et al., 2008).

It has been suggested that, in major food webs, such as that of the Baltic Sea, BMAA may be transferred from cyanobacteria to zooplankton, and bioaccumulated in fish species feeding on the sea sediment and in mussels and oysters filtering the seawater (Jonasson et al., 2010). The same pattern of BMAA bioaccumulation, with higher levels of BMAA in invertebrates and aquatic animals living near the bottom sediment, has also been reported in the subtropical marine environment in Florida Bay, USA (Brand et al., 2010).

The high amounts of BMAA found in mussels harvested along the Swedish west coast were surprising, since this area does not experience the same pronounced cyanobacterial blooms as do the typical cyanobacterial bloom-forming areas in the Baltic Proper (Boesch et al., 2008). This suggests that some other organism(s) may produce BMAA.

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In a recent publication, Jiang et al. (2014) show that BMAA is not exclusively produced by cyanobacteria. In this study BMAA was detected in six axenic cultures of diatoms and diatom containing field samples collected from the Swedish west coast. In addition and unlike cyanobacteria, diatoms are able to produce BMAA in successive generations (Jiang et al., 2014). Although dinoflagellates are suggested in this work to be BMAA producers, the hypothesis was not confirmed (Jiang et al., 2014).

Diatoms and dinoflagellates are usually the dominant phytoplankton groups and together are the foremost contributors to aquatic primary production (Armbrust, 2009). In addition, they have complementary adaptive ecologies, explaining their global niche partitioning in the turbulence/nutrient environment and onshore–offshore gradient (Smayda, 2002). Both of these phytoplankton groups are already known to include species that can produce toxic or noxious substances, or that have a nuisance impact on other aquatic organisms, the so-called harmful algal blooms (Smayda, 1997). However, of the 4000 marine planktonic microalgae species, just 200 can be harmful, and only approximately 80 of these (mainly dinoflagellates) have the potential to produce toxins (Smayda and Reynolds, 2003; Zingone and Oksfeldt Enevoldsen, 2000).

To investigate whether or not dinoflagellates can produce BMAA and to better understand the ecological availability of BMAA in field populations of diatoms and dinoflagellates, two Portuguese transitional water bodies (i.e., Ria de Aveiro and Ria Formosa) were investigated. Coastal areas are considered among the world's most productive aquatic systems, supporting dense plankton communities, constituting an ecosystem interface between continental and marine environments, receiving biogeochemical input from terrestrial, freshwater, and marine areas; including the transitional water bodies such as coastal lagoons that occupy 13% of the world's coastline (Alongi, 1998; Barnes, 1980; Nixon, 1982).

Both Ria de Aveiro and Ria Formosa are naturally inhabited year round by diatoms, including the BMAA producers (i.e. *Thalassiosira* spp., *Navicula* spp., and *Skeletonema* spp.) reported by Jiang et al. (2014), and dinoflagellate species (Cerejo, 2006; Loureiro et al., 2006; Vale and Sampayo, 2003). One important dinoflagellate species in terms of abundance, reaching high densities in the summer, is the well-known paralytic shellfish toxin (PST)-producer *Gymnodinium catenatum*, which re-emerged on the Portuguese coast in 2005 after a 10-year absence (Moita et al., 2006).

The present study determines the BMAA production by laboratory-grown *G. catenatum* cells and the possible BMAA transfer to the local cockle (*Cerastoderma edule*) population in each system. In addition, we also study the potential relationship between the BMAA and PSTs levels bioaccumulated in the cockles during the succession of *G. catenatum* blooms. For that purpose, samples of *C. edule* were taken during the 2009 *G. catenatum* bloom season from both Ria de Aveiro and Ria Formosa.

2. Materials and methods

2.1. Sampling area description

Ria de Aveiro is a shallow "bar-built" lagoon situated on the north-west Atlantic coast of Portugal, 45 km long and 10 km wide (Fig. 1). The freshwater input (approximately 1.8×10^6 m^3 during a tidal cycle of a total of 34.6×10^6 m^3 in a minimum neap tide) (Dias, 2001) comes mainly from two rivers, the Antuã River (5 m^3/s average flow) and the Vouga River (50 m^3/s average flow) (Dias et al., 1999). The only connection to the Atlantic Ocean is through a narrow channel 1.3 km long, 350 m wide, and 20 m deep. This channel makes the circulation in the lagoon essentially dominated by tidal forcing, due to the combined effects of freshwater discharge and tidal penetration, which in turn creates longitudinal gradients of salinity and temperature of 0–36 psu and 8.5–24.7 °C, respectively (Moreira et al., 1993).

Ria Formosa, on the south Portuguese coast (Fig. 1), is a barrier island system that communicates with the oceanic waters via several inlets. It is 55 km long covering an area of 160 km^2, a third of which is intertidal, with an average depth of 3.5 m (Falcão and Vale, 1990). There is no major freshwater input, leading to salinity and temperature gradients of 13–36.5 psu and 12–27 °C, respectively (Newton and Mudge, 2003).

2.2. Sampling

Samples of seawater and of cockles (*C. edule*) were collected in 2009 from Ria de Aveiro and Ria Formosa and obtained within the national monitoring program for marine toxins. Surface seawater samples were taken one hour before high tide and preserved in 2% formalin solution. *G. catenatum* was identified and enumerated in 50-mL subsamples by means of the Utermöhl technique (Hasle, 1978), using a Zeiss IM35 inverted microscope equipped with phase contrast optics and bright field illumination. Nine composite samples of *C. edule* were collected during low tide from natural shellfish beds in Ria de Aveiro between September and November 2009, while ten composite samples of *C. edule* were taken in Ria Formosa between May and November 2009. The cockles were cleaned externally with fresh water and then opened, and all their soft tissues were removed from the shell, rinsed, and drained. Each composite sample represented 30 individuals, the soft tissues of which were blended, homogenized, and stored at −18 °C until required for BMAA and PST extraction and analysis.

2.3. G. catenatum cultivation

G. catenatum culture was obtained from the algal library at the Center of Oceanography of the Faculty of Sciences, University of Lisbon. *G. catenatum* strain IO.13.20, used for this study, was originally isolated from two planktonic vegetative cells in a chain, which were collected in Sesimbra, Portugal (38°25′52″N, 9°9′52″W). *G. catenatum* was cultured in 2 L of GSe medium (Doblin et al., 1999) at 28 psu, 18 °C, and 100 μE m^{-2} s^{-1} using a 12:12 h light:dark cycle. *G. catenatum* cells were harvested by means of light vacuum filtration onto GF/C filters (Whatman, Maidstone, UK).

2.4. BMAA sample extraction

Cockles (*C. edule*) were sampled near Askö Marine Research Station (Stockholm University, Sweden) in May 2012 and used as a negative control and as a matrix for standard curve preparation for *C. edule* quantification. To quantify BMAA in the *G. catenatum* culture, a standard curve was prepared using *Spirulina* powder (Go For Life AB, Stockholm, Sweden), which was also used as a negative control. The cockles from Askö and *Spirulina* powder could be used as a matrix and a negative control since they did not contain detectable BMAA. Both standard curves had eight concentration points (i.e., 0.05, 0.1, 0.2, 0.8, 1.6, 3, 12, and 18 ng), prepared in triplicate in 2 mg (dry weight) of each matrix. Standards were added to the blank matrices just before derivatization.

Protein-associated BMAA of *G. catenatum* was extracted using our previously described procedure (Jiang et al., 2014) with the following small alterations. Triplicate samples (2-mg dry weight each) were dissolved in 1 mL of 20% methanol (20/80 methanol/water, v/v) and then lysed using sonication for 10 min at 70% efficiency (Sonopuls Model HD 2070 ultrasonic homogenizer; Bandelin Electronic, Berlin, Germany). Samples were kept in an ice-water bath throughout the procedure to prevent protein degradation. The samples were then centrifuged at 4100 × *g* for 20 min at 4 °C, and the