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BMAA Toxicological Assessment Using Marine Invertebrates as Targets

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Abstract

BMAA is a potential neurotoxin, related to neurodegenerative diseases (ALS/PDC and Alzheimer). Hypothesis that BMAA can be accumulated by some marine invertebrates has been formulated. Since it was recently discovered that in Portuguese estuaries various strains of cyanobacteria produce BMAA, the aim of this study was to obtain data about the potential transfer of the neurotoxin from cyanobacteria to marine invertebrates, which are part of the human trophic chain.

For that *Mytilus galloprovincialis* (mussel) and *Gibbula umbilicalis* (sea snail) were used for a bioaccumulation experiment. They are common organisms in Portuguese coastal ecosystems and present different ways of feeding. The collection of these species was done in a beach at Matosinhos, Portugal. These mussels and sea snails were, then, placed in aerated 20 L aquariums for acclimation during one week. Mussels were exposed to different concentrations of BMAA standard for 24 and 48 h. The results showed that mussels were able to accumulate BMAA, and the uptake rate was higher in the first 24 h.

Afterwards, mussels and sea snails were fed with cyanobacteria strains and collected at different times of exposure, ranging from 4 to 15 days of feeding and 10 days of depuration. The samples were acid digested and enzymatically digested (pepsin, trypsin and chymotrypsin) and analyzed by LC-MS/MS, with or without derivatization. The maximum concentration of BMAA in mussels was observed after 10 days of feeding with 41,92 ± 15,13 µg/g measured after acid digestion, meaning that there was bioaccumulation. After 10 days of depuration, BMAA was still detected in a concentration of 15,29 ± 0,66 µg/g. Samples digested with enzymes showed lower BMAA concentrations, as expected, but followed the same pattern of accumulation during the feeding period, followed by a decrease in BMAA concentration during the depuration period. The fact that digestive enzymes allow the release of BMAA, propose a possible way of transfer of BMAA through food chain. Regarding the sea snails, no BMAA could be measured in this organism.

Metabolic effects of BMAA were studied in cells of gills and hepatopancreas isolated from mussels fed with cyanobacteria. Inhibition of metabolic activity was observed after 20 h of cell growth, where the cells presented a lower growth rate for longer periods of feeding. Part of this inhibition of metabolic activity was considered to be due to BMAA.

With this work it was possible to see that BMAA bioaccumulation can occur in some marine invertebrates and has the potential to be transferred through the trophic chain.

Resumo

A BMAA é uma potencial neurotoxina, relacionada com doenças neurodegenerativas (esclerose lateral amiotrófica/coocorrência com Parkinson e demência e Alzheimer). Tem sido sugerido que a BMAA pode ser acumulada por alguns invertebrados marinhos. Foi recentemente descoberto que várias estirpes de cianobactérias de estuários Portugueses são capazes de produzir BMAA.

O objetivo deste estudo foi obter dados relativos à potencial transferência da neurotoxina de cianobactérias para invertebrados marinhos, que fazem parte da cadeia trófica humana.

Para tal, *Mytilus galloprovincialis* (mexilhão) e *Gibbula umbilicalis* (caracóis do mar) foram utilizados para uma experiência de bioacumulação. Estes organismos são comuns nos ecossistemas costeiros portugueses e apresentam mecanismos de alimentação diferentes. A recolha destes organismos foi realizada na praia de Matosinhos, em Portugal. Depois, estes mexilhões e caracóis do mar foram colocados em aquários de 20 L com arejamento, para aclimatação, durante uma semana. Os mexilhões foram expostos a diferentes concentrações de padrão de BMAA durante 24 e 48 h. Os resultados mostraram que os mexilhões eram capazes de acumular BMAA e que a taxa de acumulação foi maior nas primeiras 24 h.

De seguida, mexilhões e caracóis do mar foram alimentados com estirpes de cianobactérias e recolhidos a diferentes tempos de exposição, sendo que variaram entre um máximo de 15 dias de alimentação e 10 dias de depuração. As amostras foram digeridas com ácido e digeridas com enzimas digestivas (pepsina, tripsina e quimotripsina) e analisadas por LC-MS/MS, com ou sem derivatização. A concentração máxima de BMAA em mexilhões foi observada ao fim de 10 dias de alimentação com 41,92 ± 15,13 µg/g, medidos após a digestão ácida, evidenciando a existência de bioacumulação. Após 10 dias de depuração, ainda foi possível detectar BMAA numa concentração de 15,29 ± 0,66 µg/g. As amostras digeridas com enzimas mostraram concentrações mais baixas de BMAA, como era esperado, mas seguindo o mesmo padrão de acumulação durante o período de alimentação, seguido pela diminuição da concentração de BMAA durante o período de depuração. O facto de as enzimas digestivas permitirem a libertação de BMAA, sugere que existe a possibilidade de transferência desta neurotoxina através da cadeia alimentar. Relativamente aos caracóis do mar, não foi possível detectar BMAA nestes organismos.

Os efeitos metabólicos da BMAA em células das brânquias e do hepatopâncreas isoladas dos mexilhões alimentados com cianobactérias, foram estudados. Foi observada inibição da atividade metabólica após 20 h de crescimento celular, sendo que as células apresentaram uma taxa de crescimento mais baixa nos períodos mais longos de alimentação. Parte desta inibição considerou-se devida à BMAA.

Com este trabalho, foi possível observar que a BMAA pode ser acumulada em alguns invertebrados marinhos e pode ser transferida através da cadeia trófica.

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List of abbreviation

AEG - *N*-(2-aminoethyl) glycine

ALS - Amyotrophic lateral sclerosis

BMAA - β-N-methylamino-L-alanine

DAB - 2,4-diaminobutyric acid

LC-MS/MS - Liquid chromatography with tandem mass spectrometry detection

LOD – Limit of detection

MN – Motor neuron

m/z – Mass-to-charge ratio

PDC - Parkinsonism dementia complex

PSP – Paralitic shellfish poisoning

PTFE – Polytetraflouroethylene

ROS – Reactive oxygen species

SOD – Superoxide dismutase

SPE – Solid-phase extraction

TIC – Total ion current

1. Introduction

1.1. Cyanobacteria

Cyanobacteria, also known as blue-green algae, can be distinguished from all other prokaryotes by their ability to carry out oxygen-producing photosynthesis (Paul, 2008). They can grow in any type of water (freshwater, brackish and marine) (Bláha et al., 2009). The cyanobacteria provide an extraordinarily wide-ranging contribution to human affairs in everyday life and are of economic importance (Bartram et al., 1999). They are important primary producers and their general nutritive value is high (Bartram et al., 1999). Due to the fact that they include nitrogen-fixing species, they contribute to soil and water fertility (Bartram et al., 1999). They are, also, used in alimentation and food production (Bartram et al., 1999). However, abundant growth of cyanobacteria in water reservoirs creates severe practical problems for water supplies (Bartram et al., 1999). There are two main factors that contribute for the increase of occurrence of these blooms: anthropogenic eutrophication, with nutrient overenrichment of waters by urban, agricultural, and industrial development; and climate change, a potent catalyst for the expansion of these blooms (elevation of temperature, increased atmospheric concentrations of carbon dioxide, elevated UV fluxes, and elevated pH values) (Paerl and Huisman, 2008; Bláha et al., 2009).

These blooms increase the turbidity of aquatic ecosystems, smothering aquatic plants and thereby suppressing important invertebrate and fish habitats (Paerl and Huisman, 2008). Die-off of blooms may deplete oxygen, killing fish (Paerl and Huisman, 2008). So the high nutrient loading, rising temperatures, enhanced stratification (reducing vertical mixing), increased residence time, and salination are all factors that promote cyanobacterial dominance in many aquatic ecosystems (Paerl and Huisman, 2008).

The occurrence of cyanobacterial mass populations can create a significant water quality problem, especially as many cyanobacterial species are capable of synthesizing a wide range of noxious compounds or potent toxins (cyanotoxins) (Sivonen and Jones, 1999).

1.2. Cyanotoxins

Cyanotoxins can be divided following two main criteria (Sivonen and Jones, 1999):

- On the basis of their mechanism of action on terrestrial vertebrates, especially mammals (hepatotoxins, neurotoxins, dermatoxins, cytotoxins and irritant toxins);
- According to their chemical structure (cyclic peptides, alkaloids or lipopolyssacharides);

Table 1 shows the main cyanotoxins, the producing cyanobacteria and their mechanism of action.

Table 1. *Cyanotoxins, the producing cyanobacteria and mechanism of action.*

Toxin	Cyanobacteria genera	Mechanism of action
Microcystins	*Microcystis*	Inhibition protein phosphatase (PP1 and PP2A)
	Anabaena	
	Plankthotrix	
Nodularin	*Nodularia*	Inhibition protein phosphatase (PP1 and PP2A)
Saxitoxins	*Anabaena*	Binding and blocking the sodium channels in neural cells
	Aphanizomenon	
	Cylindrospermopsis	
	Lyngbya	
Anatoxins	*Anabaena*	Binding irreversibly to the nicotinic acetylcholine receptors
	Aphanizomenon	
	Cylindrospermopsis	
	Plankthotrix	

	Oscillatoria	
	Microcystis	
Anatoxin-a (s)	*Anabaena*	Inhibition of acetylcholinesterase activity
Cylindrospermopsin	*Cylindrospermopsis*	Inhibitor of protein biosynthesis cytogenetic damage on DNA
	Aphanizomenon	
	Umezakia	
	Raphidiopsis	
	Anabaena	
Lipopolysaccarides	Cyanobacteria in general	Irritant; causes inflammation in exposed tissues
BMAA	Cyanobacteria in general	Binding to the glutamate receptors, like NMDA, AMPA and mGluR

1.3. History and relevance of BMAA

β-N-methylamino-L-alanine (BMAA) is a neurotoxic non-protein amino acid produced naturally by cyanobacteria found in freshwater, marine and terrestrial ecosystems (NIEHS, 2008). This toxin was first isolated by Vega and Bell (1967) from cycad seeds, in Guam (Pacific Ocean) (Cohen, 2012). Among Chamorro people of Guam the incidence of neurodegenerative illness (mostly Amyotrophic Lateral Sclerosis/Parkinsonism Dementia Complex) was one of the highest in the world (Cohen, 2012). Amyotrophic Lateral Sclerosis (ALS) is a debilitating and fatal neuromuscular disease and approximately 8–10% of all cases are familial, about half of which are characterized by superoxide dismutase mutation (SOD-1). The other 90–92% of ALS cases occurs sporadically, with no known familial history (Banack et al., 2010b). At present, there is no known cause for sporadic ALS, though a number of environmental compounds have been implicated as potential etiological agents (Banack et al., 2010b). BMAA has been linked to the high incidence of the neurodegenerative disease amyotrophic lateral sclerosis/parkinsonism-

dementia complex (ALS/PDC) among the indigenous Chamorro people on the islands of Guam (Cox et al., 2003). BMAA was present throughout cycad tissues, but was particularly abundant in cycad seeds and immature pollen (Banack and Cox, 2003). The cycad seeds are washed and used as flour by the indigenous Chamorro people, and they are also used as food by wild animals including flying foxes, pigs and deer (Cohen, 2012). The suggested link between BMAA and ALS/PDC in Guam is because of (Pablo, 2009):

- BMAA is produced by cyanobacteria, including endosymbiotic species of *Nostoc* (a genus of cyanobacteria) found in cycad roots;
- BMAA occurs in higher amounts in cycad flour than was previously suspected because of its occurrence in the protein fraction (i.e., BMAA binds to a protein);
- BMAA can be biomagnified in animals which forage on cycad seeds and are subsequently consumed by the Chamorro people.

The biomagnification was reported by Murch et al. (2004b), where they observed the biomagnification at Guam ecosystem. In this study, BMAA was found as protein-bound in symbiont-cyanobacteria (namely *Nostoc*) of *Cycas micronesica* (between 2 and 738 µg/g), in cycad flour (between 49 and 168 µg/g) and in flying foxes (between 2 and 146 µg/g).

The same authors found that BMAA, both as a free amino acid and as a protein-bound amino acid, occurs in the brain tissues of Chamorro patients who died from ALS/PDC (Murch et al., 2004a). Thus, a trophic chain where BMAA was present from primary producers to the consumers was found.

BMAA was, also, detected in the brains of Canadian patients with Alzheimer's disease but not in control patients without neurological disease (Murch et al., 2004a). Another interesting finding was made by Pablo et al. (2009), that showed the presence of BMAA in the brains of USA patients, but not in those of patients with Huntington's disease (a genetic neurodegenerative disease) (Pablo et al., 2009). The negative screen for BMAA in Huntington's disease, suggests that BMAA does not occur as a byproduct of neurodegenerative disease (Pablo et al., 2009; Banack et al., 2010b).

The fact that it was reported that all known groups of cyanobacteria can produce BMAA allowed more studies of bioaccumulation in aquatic environment (Cox et al., 2005), especially in marine ecosystems, where many organisms from different trophic levels presented BMAA (Brand et al., 2010; Jonasson et al., 2010).

1.4. Physical and chemical properties of BMAA

BMAA is a polar, non-protein basic amino acid. Some chemical and physical properties are shown in table 2.

Table 2. *Chemical and physical properties of BMAA (adapted from NIEHS, 2008).*

Synonyms:	L-β-Methylaminoalanine β-methylamino-L-alanine (+)-L-β-N-Methyl-α,β-diaminopropionic acid L-α-amino-β-methylamino propionic acid L-MeDAP (methylated α,β-diaminopropionic acid)
IUPAC:	(2S)-2-amino-3-(methylamino)propanoic acid
CAS Registry No:	15920-93-1
Molecular formula:	$C_4H_{10}N_2O_2$
Molecular weight:	118,1344 g/mol or Da

BMAA is an amino acid and can be found as *L*- and *D*-α-amino-β-methylaminopropionic (Vega et al., 1968). Vega and colleagues (1968) reported that the *D*- isomer was not toxic when injected in chicks and young rats in comparison with the *L*-isomer that produced a toxic effect. The last isomer was the one isolated in seeds of *Cycas circinalis* (Vega and Bell, 1967).

Many different isomers of BMAA have been reported and when the objective of the study is to detect and quantify this neurotoxin, these isomers have to be considered. From these isomers the most reported in literature are DAB (2,4-diaminobutyric acid) and AEG (*N*-(2-aminoethyl) glycine), that are molecular isomers. DAB is believed to be a hepatotoxic and neurotoxic non-protein amino acid that is found in many prokaryotic and eukaryotic organisms (Jiang et al., 2012). AEG is a backbone for peptide nucleic acids and its effects have not yet been discovered (Banack et al., 2012). In figure 1 it's show the structure of BMAA, DAB and AEG.

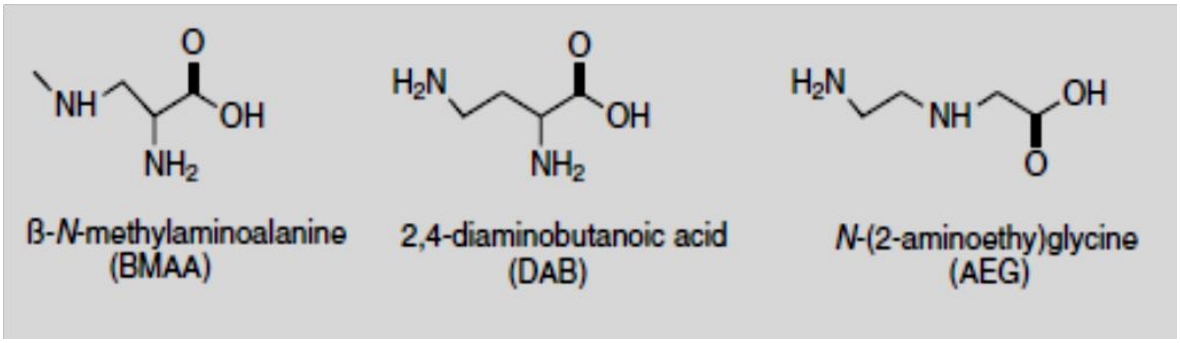

Figure 1. *Structure of BMAA and selected isomeric compounds (DAB and AEG) (adapted from Jiang et al., 2012).*

1.5. Mechanism of action of BMAA

The BMAA that is bound to proteins within the body can slowly be released into cerebral tissues in the free form when these proteins are metabolized over time, acting as an endogenous neurotoxic reservoir (Murch et al., 2004a; Cox et al., 2005). BMAA has been found to induce selective motor neuron (MN) loss in dissociated mixed spinal cord cultures at concentrations of approximately 30 μM, significantly lower than those previously found to induce widespread neuronal degeneration and supporting the hypothesis that BMAA may contribute to the selective MN loss in ALS/PDC (Rao et al., 2006; Lobner et al., 2007). One mechanism of action of this toxin is excitotoxicity. This type of toxicity is characterized by malfunctions in EAAs (excitatory amino acids – neurotransmitters within the nervous system) that can lead to neurons being damage and fatally compromised (Chiu et al., 2011). Excitotoxic cell death involves prolonged depolarization of neurons, changes in intracellular calcium concentrations, and the activation of enzymatic and nuclear mechanism of cell death (Doble, 1999). AMPA (quistalate/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA (N-methyl-D-apartate) and mGluR (metabolic glutamate receptors) are the main EAA receptors, all of which are activated by glutamate and similar substances (Chiu et al., 2011). Many studies shown that excitotoxicity is a main player in neurodegenerative disease, because there is an increased level of glutamate found in the cerebrospinal fluid of ALS patients (Majoor-Krakauer et al., 2003; Shaw, 2005). So, when BMAA is orally consumed, 80% of ingested BMAA passes from the gut into the blood stream (Chiu et al., 2011). BMAA then crosses the blood-brain barrier via large neutral amino acid carriers (Chiu et al., 2011). It is known that physiological concentrations of bicarbonate ions react with BMAA to form β-carbamate, and in this form BMAA can compete in binding to various glutamate receptors, like NMDA receptors, AMPA receptors and mGluR (Banack et al., 2010b; Rao et al., 2006). The activation of this receptors leads to shifts in cellular ion concentrations

resulting in increases in Na^+ and Ca^{2+}, and a decrease in K^+ concentrations, and also causes the cell to become depolarized leading to permeabilisation of the cell membrane, resulting in the release of noradrenalin (Chiu et al., 2011). BMAA also inhibits the cysteine/glutamate antiporter system Xc^-, preventing the uptake of cysteine, resulting in glutathione depletion, which contributes to increases in oxidative stress (Liu et al., 2009). At the same time the system Xc^- increases the release of glutamate from the cell (Liu et al., 2009). Glutamate can then bind to glutamate receptors increasing damage by excitotoxicity. Increases in intracellular Ca^{2+} concentrations disrupt normal mitochondrial function leading to the release of ROS (reactive oxygen species) into the cytoplasm, thereby contributing to the observed increases in oxidative stress (Lobner et al., 2007). In addition, cytochrome-c is released from mitochondria resulting in the induction of apoptosis (Chiu et al., 2011).

Beside this mechanism of action, it has also been suggested that BMAA can be incorporated into proteins and subsequently lead to protein misfolding (Banack et al., 2010b). The tRNA synthetase enzyme for the amino acid serine mistakenly picks up BMAA and incorporates it into proteins *in vitro* (Holtcamp, 2012). Because of this misincorporation and consequent occurrence of mistakes in translation, the hydrophobic parts of the protein end up exposed. These parts adhere to other misfolded proteins, forming “aggregates”, a characteristic of neurodegenerative diseases. The formation of small aggregates seeds the formation of larger, more toxic aggregates in a sort of chain reaction that prevents the cells from functioning effectively (Holtcamp, 2012).

These multiple mechanisms of BMAA toxicity may account for its ability to induce complex neurodegenerative diseases (Banack et al., 2010b).

1.6. Analysis of BMAA

Concerning the analysis of BMAA, different methods have been used. High performance liquid chromatography with fluorescence detection (HPLC-FLD) (Cox et al., 2005; Banack et al., 2007; Johnson et al., 2008; Metcalf et al., 2008; Eriksson et al., 2009; Cianca et al., 2012a), gas chromatography with mass spectrometry detection (GC-MS) (Esterhuizen and Downing, 2008), capillary electrophoresis (CE) (Baptista et al., 2011), amino acid analyzer (Banack et al., 2007), nuclear magnetic resonance spectroscopy (Moura et al., 2009), liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) (Kubo et al., 2008; Rosén et al., 2008; Faassen et al., 2009; Banack et al., 2010a; Kruger et al., 2010; Li et al., 2010; Spácil et al, 2010; Jonasson et al., 2010), and

more recently commercial enzyme linked immunosorbent assay (ELISA) (Faassen et al., 2013), have been reported for the measurement of BMAA. It was hypothesized that different analytical methods for the determination of BMAA deviate in their results (Faassen et al., 2012). For example, high BMAA concentrations and high percentages of positives samples were found only in those studies that had used HPLC-FLD (Cox et al., 2005; Metcalf et al., 2008), GC-MS (Esterhuizen and Downing, 2008) or CE (Baptista et al., 2011). On the other hand, studies that had used LC-MS/MS for quantification either did not detect BMAA, or reported lower BMAA concentrations (Rosén and Hellenas, 2008; Faassen et al., 2009; Jonasson et al., 2010; Kruger et al., 2010). Faassen et al. (2012) concluded, in their study, that HPLC-FLD overestimated BMAA concentrations in some cyanobacterial samples due to its low selectivity. They also recommended to only use selective and sensitive analytical methods like LC-MS/MS (with derivatization or not) for BMAA analysis (Banack et al., 2010a; Faassen et al., 2012).

Another difference that occurs in these studies is the use (or not) of derivatization. Because BMAA has no chromophore, it needs to be derivatized in order to be detected by fluorescence (Cianca et al., 2012b). Derivatization is also used with LC-MS/MS, because the electrospray ion source (ESI) desorption of charged molecules occurs from the surface, meaning that sensitivity is higher for compounds with a greater concentration at the surface of the droplet, thus the more lipophilic ones (Banack et al., 2010a). The derivatized compounds are more lipophilic compared to underivatized ones and also have higher molecular weight, being easier to detect (Banack et al., 2010a).

However, some concerns are given to the use of derivatization. This step in the sample preparation can interfere in the results, since there is a theoretical risk that a compound similar to BMAA would react to give a BMAA-derivative during the derivatization procedure (Rosén and Hellenas, 2008). So, many investigators have used methods of analysis of BMAA without using derivatization (Kubo et al., 2008; Rosén and Hellenas, 2008; Faassen et al., 2009; Faassen et al., 2012).

1.7. Bioaccumulation of BMAA and ways of exposure

The bioaccumulation of cyanotoxins by aquatic organisms is widely described in literature. Several of these studies have shown that mollusks are the most frequent organisms that are able to accumulate cyanotoxins, like mycrocystin-LR (Vasconcelos, 1995; Pires et al., 2004), PSP (Pereira et al., 2004), nodularin (Karlsson et al., 2003), cylindrospermopsin (Saker et al., 2004) and anatoxin-a (Osswald et al., 2008).

Since BMAA was found to be synthesized from cyanobacteria and is not a byproduct of neurodegeneration (Pablo et al., 2009), the identification of BMAA, not only in the brains of ALS/PDC patients who died in Guam, but also of patients who had died of Alzheimer's disease in Canada and USA, suggests that exposure to cyanobacterial neurotoxins exists outside Guam and that BMAA may be more widely distributed than previously supposed (Murch et al., 2004a; Bell, 2009).

This was further evidenced by the finding that BMAA can be produced by all known groups of cyanobacteria, including cyanobacterial symbionts and free-living cyanobacteria (terrestrial, as well as freshwater, brackish, and marine) (Cox et al., 2005). In British waterbodies, Metcalf et al. (2008) reported the presence of BMAA in samples of cyanobacteria from different blooms. They also showed that BMAA can co-occur with others cyanotoxins, including microcystins, anatoxin-a, nodularin and saxitoxin (Metcalf et al., 2008). In Portuguese estuaries, free and protein bound BMAA were found in cyanobacteria (Cianca et al., 2012b).

Esterhuizen et al. (2011) showed that free BMAA can be released during the collapse of cyanobacterial blooms. This process combined with cellular turnover creates a latent source of the non-protein amino acid for bioaccumulation and biomagnification in aquatic ecosystems. Following accumulation of free BMAA, protein association was observed which suggests a potential route for human exposure to BMAA (Murch et al., 2004b; Esterhuizen et al., 2011). As it was early recognized, BMAA can be biomagnified in animals on Guam ecosystem (Cox et al., 2003; Banack et al., 2006).The evidence of the widespread occurrence of BMAA, suggest that bioaccumulation of BMAA could occur within other organisms outside of Guam (Cox et al., 2005).

Several studies have reported the presence of BMAA in animals in marine environment, evidencing that some transfer exist through trophic chain. For example, Brand et al. (2010) found that many crustaceans appear to have high concentrations of BMAA (7000 µg/g) in South Florida, where it is usual to occur many blooms of cyanobacteria.

In the Baltic Sea was found that BMAA is biosynthesized by cyanobacteria (Jonasson et al., 2010). Like in South Florida, BMAA was also found in organisms of higher trophic levels that directly or indirectly feed on cyanobacteria, such as zooplankton (87 ng/g) and various vertebrates (fish) and invertebrates (mussels, oysters), where concentrations ranging from 10 ng/g to 1290 ng/g were found (Jonasson et al., 2010).

It was also reported levels of BMAA in feathers of Lesser Flamingo (77 ng/g), which consume cyanobacteria as a major part of their diet, in Kenyan lakes (Metcalf et al., 2013).

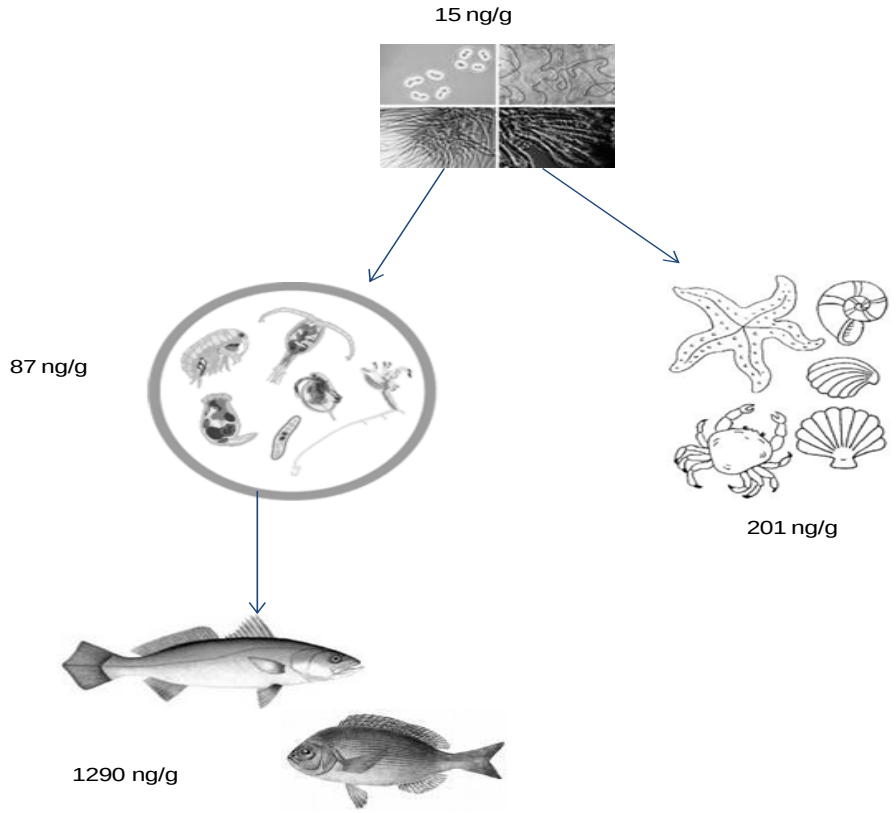

Figure 2. *BMAA transfer in an aquatic trophic chain (adapted from Jonasson et al., 2010).*

Therefore, human population may potentially be exposed to BMAA from water sources containing cyanobacteria blooms and consumption of fish or animals that have been exposed to BMAA (NIEHS, 2008).

Another postulated route of cyanotoxin exposure is inhalation of aerosolized toxins (Banack et al., 2010b). There are many examples of toxin aerosolization leading to human illness (Stommel et al., 2013). An example is the brevetoxin that can be aerosolize through wave action of marine waters, causing asthma like syndrome (Fleming et al., 2007). Other example is the generation of aerosolized microcystin, making inhalation a potential route of exposure (Backer et al., 2008). Consequently, Stommel et al. (2013) hypothesize that white caps on waterbodies containing cyanobacterial blooms and cyanobacteria present on cooling towers, could result in aerosolization of BMAA.

1.8. Objectives

The objective of this work was to obtain data about the potential transfer of BMAA from cyanobacteria to marine invertebrates, which are part of the human trophic chain.

For this, two different species of marine invertebrates, with different ways of feeding were chosen. *Mytilus galloprovincialis* (a filter feeder) and *Gibbula umbilicalis* (a grazer) were fed with cyanobacteria, growing in laboratory, that had been isolated from Portuguese northern estuaries, to determine BMAA uptake, in a bioaccumulation experiment in which different periods of feeding and depuration were tested.

2. Material and Methods

2.1. Species ecology

M. galloprovincialis (blue mussel or Mediterranean mussel) is native to the Mediterranean coast and the Black and Adriatic Seas. This mussel is dark blue or brown to almost black. The two shells are equal and nearly quadrangular. The outside is black-violet colored. On one side the rim of the shell ends with a pointed and slightly bent umbo while on the other side is rounded, although the shape of the shell can vary from region to region.

In its native range, *M. galloprovincialis* can be found from exposed rocky outer coasts to sandy bottoms (Ceccherelli and Rossi, 1984). It typically requires rocky coastlines with a high rate of water flow.

Regarding the nutrition, *M. galloprovincialis* is a filter-feeding bivalve that eats a wide range of planktonic organisms. This species prefers fast moving water that is free of sediment and thrives in regions where nutrient-rich upwelling occurs. This mussel is an important component of estuarine and marine food webs and because it is a sessile filter feeder, it may be exposed to high density of cyanobacteria and their toxins (Osswald et al., 2008).

G. umbilicalis is found on the European coasts of the Atlantic Ocean. This sea snail belongs to the *Trochidae* family. It is found from the upper shore into the sublitoral on sheltered rocky shores and is tolerant of emersion and brackish waters. It is characterized by having a small top shell, broader, rounded and flatter. It also has a large round umbilicus (a deep hole on the underside of the shell). Concerning other features, it is greenish-grey in color and has broad diagonal stripes of reddish-purple. They live as algae grazers and detritus eaters. These species are not normally used in bioaccumulation studies. However, recently, it was shown that levels of tetrodotoxin were detected in *G. umbilicalis* (Silva et al., 2012; Silva et al., 2013).

These two species are used as food by other animals (including humans), providing a way of exposure to cyanotoxins, namely BMAA, through food chain.

2.2. Cyanobacteria culture growth

Previous studies regarding BMAA production and cyanobacteria characterization served as the basis for the choice of species (Cianca et al., 2012b; Brito et al., 2012). The cyanobacteria were isolated as described in Lopes et al. (2010) and kept growing at LEGE laboratory (CIIMAR) as part of the culture collection. The selected strains of cyanobacteria were grown in BG_{11} (Rippka et al., 1979) and MN (Waterbury and Stanier, 1978) and Z8 (Kotai, 1972) growth media for two weeks, at 25ºC of temperature and under light intensity of 20,8 – 27,4x10^{-6} E m^{-2} s^{-1} and a light/dark cycle of 14/10 h (figure 3). Some modifications were introduced in the culture media, namely in $BG_{11}{}^{15N}$ (a variant of the BG_{11} medium), in which labeled ^{15}N was delivered as $^{15}NH_4Cl$ (table 3).

Table 3. *Cultured cyanobacteria strains.*

LEGE code	Strain	Medium	Environment
#06077	*Nostoc* sp.	MN	Estuary
#06077	*Nostoc* sp.	$BG_{11}{}^{15N}$	Estuary
#06079	*Synechocystis salina*	$BG_{11}{}^{15N}$	Estuary
#06079	*Synechocystis salina*	Z8	Estuary

The cells were grown to obtain enough biomass to feed the animals (10^5 cells/mL). At the end of the experiment the cells were harvested by centrifugation. Afterwards, all samples were lyophilized and stored at -20ºC.

Figure 3. *Cyanobacteria culture room.*

2.3. Bioaccumulation experiment

The collection of *M. galloprovincialis* and *G. umbilicalis* was done in a beach at Matosinhos, Portugal (figure 4). These mussels and sea snails were, then, placed in aquariums in BOGA (Biotério de Organismos Aquáticos) at CIIMAR for acclimation during one week. The aquariums had capacity for 20 L and had aeration. These were filled with 3 L of marine water and every two days the water was changed. Some sea snails and mussels were also collected from the beach and frozen (without any treatment) for subsequent analysis.

Figure 4. *Sampling site of M. galloprovincialis and G. umbilicalis at Matosinhos.*

2.3.1. Mussels exposed to BMAA standard

M. galloprovincialis were exposed to BMAA standard (Sigma – Aldrich) with concentrations of 1, 2,5 and 5 mg/L for periods of 24 h and 48 h. The BMAA standards were dissolved in the seawater and four individuals were exposed to each concentration. The mussels were not fed. One death was observed during this experiment.

2.3.2. Mussels fed with cyanobacteria

Approximately sixty individuals kept in 20 L aquariums and aerated, were fed with *S. salina,* growing in Z8 medium*,* every two days (figure 5A). The water was changed every two days. The mussels were collected after 4, 8 and 10 days of feeding and at the end of the experiment after allowing 10 days of depuration. No deaths were observed during this experiment.

Control mussels, kept in a separate aquarium, were fed with the green algae *Chlorella sp*. grown as described in 2.2.

2.3.3. Sea snails fed with cyanobacteria

G. umbilicalis were placed in 20 L aquariums and aerated (120 organisms in each), as described before, and fed with *S. salina,* growing in $BG_{11}{}^{15}N$ culture media*,* and *Nostoc* sp., one growing in MN culture medium and the other one in $BG_{11}{}^{15}N$, every two days (figure 5B)*.* The water was also changed every two days.

Chlorella sp. was used as control. After 5, 10 and 15 days of feeding, and 5 days of depuration, the sea snails were collected. Five deaths were observed during this experiment.

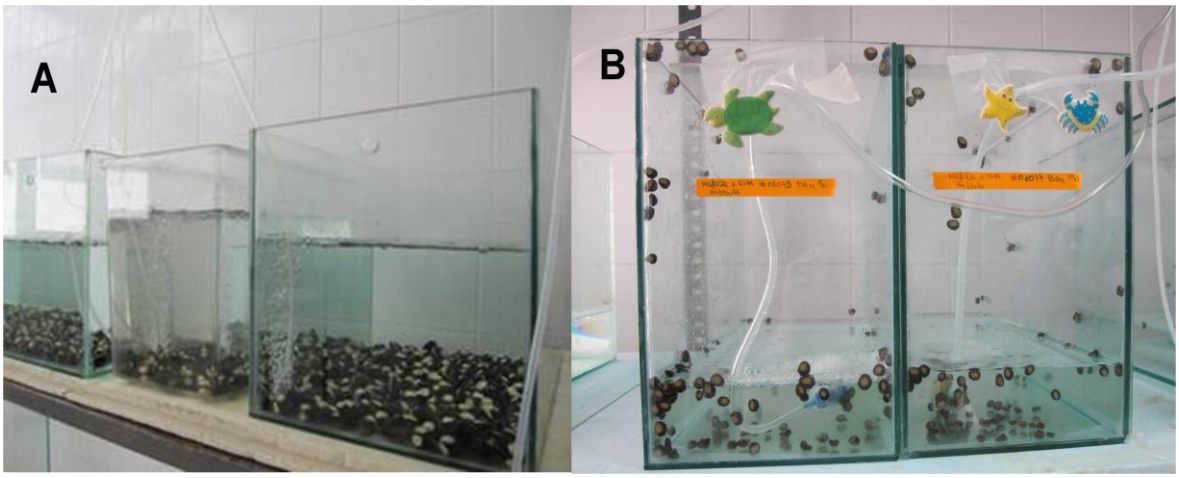

Figure 5. *Aquariums with M. galloprovincialis (A) and G. umbilicalis (B).*

2.4. Acid microwave-assisted extraction

The soft parts of the body of the mollusks were removed from their shells, weighted and digested.

Usually, the hydrolysis step to extract BMAA uses 6 M HCl at 110ºC, with times of digestion varying from 12 to 24 h (Faassen et al., 2009; Jonasson et al., 2010). However, Baptista et al. (2011) suggested this step could be performed using a microwave system. The main advantages of the microwave-assisted digestion are the shorter time of the procedure, the possibility to control the temperature and/or the pressure inside the microwave vessels, and the possibility to process a larger number of samples at the same time (Baptista et al., 2011).

To optimize the microwave-assisted hydrolysis of BMAA three different programs were tested. Time, temperature and power of the hydrolysis were varied as shown in table 4. For that, 1 mg/L BMAA standard (1 replica) was transferred to closed PTFE vessels and digested using a high-pressure microwave system (Milestone-Ethos1).

Table 4. *Programs used for the optimization of the microwave-assisted hydrolysis.*

Power (W)	**Temperature (ºC)**	**Time (min)**
300+400+500	>50	5+5+5
300	>50	10
<1000	90	20

Mussels exposed to BMAA standard and mussels and sea snails fed with cyanobacteria and cyanobacteria biomass were digested with 2 mL of 6 M HCl, for 20 min at 90ºC, 10 mg per sample. Three replicas per sample were digested. The complete dissolution of the biomass was achieved as verified by the clarity of the digest. Afterwards the samples were evaporated under a low flux of nitrogen.

2.5. Enzymatic digestion

Mussels and sea snails fed with cyanobacteria were also digested with digestive enzymes, namely proteases: pepsin, trypsin and chymotrypsin.

The enzymatic digestion was performed according to Bauer et al. (2003), with minor modifications. The reagents used for this method were prepared as follows:

- Pepsin/HCl solution: 0,8 g of pepsin was dissolved in 500 mL of HCl solution (4,25 mL HCl and water);
- Trypsin/Chymotrypsin solution: 0,9 g of each enzyme were dissolved in a pH 7,8 phosphate buffer (560 mL of Na_2HPO_4 0,165 mol/L and 440 mL of $KH_2PO_4.12H_2O$ 0,165 mol/L)
- HCl/Acetic acid solution: HCl (1 mol/L) and acetic acid (1 mol/L) were combined in a ratio of 5:2.

Samples were digested with pepsin, or with trypsin/chymotrypsin. For the pepsin digestion the samples were incubated in 50 mL tubes with 8,5 mL of pepsin/HCl solution.

For the trypsin/chymotrypsin digestion the samples were incubated in 50 mL tubes with 10 mL of trypsin/chymotrypsin solution. At the end of the digestion the solution was neutralized by addition of 1,6 mL of HCl/acetic acid solution.

Three replicas of 100 mg samples were made for each digestion. The samples were incubated in a water bath, at 37ºC, for 2 h with constant stirring. After this, the samples were centrifuged and the supernatant was collected. All samples were then pre-concentrated using solid-phase extraction (SPE).

2.6. SPE

Mussels and sea snails digested with enzymes and sea snails digested with HCl were pre-concentrated by solid-phase extraction using a cartridge Isolute HCX-3, 100 mg (Biotage) and the cleanup procedure described by Spácil et al (2010). The solid phase column was pre-conditioned with 1 mL MeOH. After that, it was conditioned with 2 mL 0,1% formic acid/H_2O.

HCl digested samples were reconstituted in 1 mL of 0,1% formic acid/H_2O and applied to the column. The enzymatically digested samples were applied to the SPE column. The washing step was done using 1 mL 0,1% formic acid/H_2O, followed by a two-step elution. The first elution step consisted of 1 mL 0,1% formic acid in 25% MeOH. The second elution step consisted of 1 mL 2% NH_4^+ in MeOH. Both eluates were evaporated using a freezdrier (-110ºC, vacuum).

2.7. LC-MS/MS analysis

2.7.1. Analysis without derivatization

Liquid Chromatography with mass detection (LC-MS/MS) without derivatization was used to measure BMAA in cyanobacteria and mussels samples. The samples were reconstituted with 20 mM HCl, before the analysis with LC-MS/MS.

Analyses were performed using a system that comprised a LC (Thermo Scientific) coupled to a MS (Thermo Scientific, LCQ Fleet) with an ESI interface. The electrospray interface was operated using the following settings: positive polarity and nebulizer, N_2 (1,5

L/min; drying gas, N_2 at 0,1 Mpa). Full-scan MS was performed from *m/z* 50 to 150 at 0,5 s^{-1} scan in the continuum mode. LC conditions were as follows: column, ZIC-HILIC (100 mm x 2,1 mm; with guard column); mobile phase of 90–60% aqueous linear gradient of acetonitrile (ACN) for 20 min, then 60% aqueous ACN maintained for 15 min; flow rate of 0,5 mL/min; column temperature, 40ºC; and CE 16,0 (collision energy) .

To obtain an analytical curve and to determine the detection limit for BMAA, standard BMAA solutions (10 µg/L to 1000 µg /L), which were prepared using 20 mM HCL as the solvent, were used (Kubo et al., 2008).

Selected specific product ions (*m/z* 102, 88, 73), all derived from the precursor ion *m/z* 119 (BMAA molecular weight ($M + H^+$)) and originated from different parts of the molecule were selected. The ion used for quantification was *m/z* 119, whereas the other two ions were used as qualitative. These products were detected in previous LC-MS/MS analysis of BMAA (Rosén and Hellenas, 2008). Excalibur V2.1 software was used to explore acquired chromatographic data. The limit of detection was 1,7 pmol/injection.

2.7.2. Analysis with derivatization

Liquid Chromatography with mass detection (LC-MS/MS) with derivatization was used to measure BMAA in cyanobacteria, sea snails and mussels samples.

Standards and samples were derivatized with AQC - 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Waters AccQ·tag ultra-kit, Milford, MA, USA). All chemicals used for the reaction were supplied with the kit and solutions were applied as follows: 20 µL of 20 mM HCl was added to redisolve the dried sample pellet, then 70 µL of borate buffer was added followed by 30 µL ACQ tag. The sample was then vortexed and analyzed within 48 h. ESI ionization was performed in positive ion detection mode. The mass analyzer used SRM scan type with recorded transitions: 459,1>119,1 (CE 30,0), DAB 459,1>188,0 (CE 38,0), AEG 459,1>214,1 (CE30,0), BMAA 459,1>258,0 (CE 30,0). Other parameters of the ESI source and the mass analyzer are listed below: capillary voltage (5500V), source temperature (450C), declustering potential (50), focusing potential (350), and entrance potential (6).

The LC system was an Acqutiy UPLC Xevo, TQ-MS with an Acquity UPLC, BEH C18, 1,7 µm, 2,1 x 100 mm column. Eluent A was 0,01% formic acid in 0,05% ammonia and water; eluent B was 0,01% formic acid in methanol. The elution program was applied as

follows: 0,0%A till 4,00 min; 99,9%A 0,1%B till 4,10 min; 45%A 45%B till 4,70 min; 100%B till 6,00 min; 99,9%A 0,1%B till end. To obtain an analytical curve and to determine the quantification limit for BMAA, standard BMAA solutions (1 µg/L to 1000 µg/L), which were prepared using 20 mM HCL as the solvent, were used. The ion used for quantification was *m/z* 119, whereas the *m/z* 258 was used as qualitative. MassLynx V4.1 software was used to explore acquired chromatographic data. The limit of detection was 0,4 pmol/injection.

2.8. Metabolic activity of cells of mussels fed with cyanobacteria

The primary cells for this experience were obtained as previously described by Louzao et al. (2010). Mussel gills and hepatopancreas, from the mussels fed with cyanobacteria, were excised, separately washed in ice-cold sea water and sliced with a bistoury in small fragments. Then, gills and hepatopancreas portions were immersed in ice-cold sea water and gently agitated with a magnetic stirrer. After washing for 15 min the slices were rinsed twice with fresh ice-cold sea water and filtered through 200 µm nylon meshes three times. Finely chopped 5-10 g of gills and hepatopancreas tissues were enzymatically dispersed with 3500 IU/mL collagenase and 1 IU/mL dispase in 20 mL of artificial sea water for 45 min at 37ºC (Louzao et al., 2010). The treatment resulted in a suspension containing the cells, which were harvested by centrifugation at 800 rpm for 5 min. 200 µL of the cell-containing suspension were seeded on 96-well plates. Three replicates of each sample were made. 20 µL of Alamar Blue (Biosource; Invitrogen Corporation), diluted 1:5, was added and fluorescence was measured using a microplate reader (Biotek synergy HT). Cells were incubated in constant shaking at room temperature, and measurements were registered at 2,5; 5 and 20 h. Fluorescence was monitored at 530-560 nm excitation wavelength and 590 nm emission wavelength.

The Alamar Blue Assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth (Ahmed et al., 1994).

The REDOX indicator exhibits both fluorescence and colorimetric change in the appropriate oxidation-reduction, is demonstrated to be minimally toxic to living cells and produces a clear, stable distinct change which is easy to interpret. Metabolic activity of the

cells being tested results in a chemical reduction. Continued metabolic activity maintains a reduced environment while inhibition of metabolic activity results in an oxidized environment. Reduction causes the REDOX indicator to change from oxidized (non-fluorescent, blue) form to reduced (fluorescent, red) form. Fluorescence is proportional to the number of metabolic active cells.

2.9. Chemicals and reagents

L- BMAA hydrochloride, its structural isomer DAB and AEG, formic acid, ammonia, HCl p.a. 37% (hydrochloric acid) were purchased from Sigma-Aldrich. The enzymes used, were purchase, also, from Sigma-Aldrich. The AccQTagfluor (AQC) reagent Kit was purchased from Waters Corporation. Acetonitrile, methanol and chromatographic-grade water was obtained from VWR. The deionized water (dH_2O) had a conductivity < 0,1 µS/cm. Alamar Blue was from Biosource^{TM}, Invitrogen Corporation.

2.10. Statistical analysis

BMAA content results are the mean values of four or three independent replicates. A *t*-test or a one-way analysis of variance (ANOVA) was performed to test for significant differences in mean concentration. Whenever significant differences were found ($p \leq 0,05$) a Tukey test was performed, using SPSS 21 Software.

The uptake rate was calculated dividing the mean concentration by the hours of exposure.

For the metabolic activity assay, the growth rate was calculated using the follow formula: ln(Cf/Ci), where ln is natural logarithm, the Cf is final concentration and Ci is initial concentration.

3. Results

3.1. Microwave-assisted extraction optimization

To optimize BMAA hydrolysis, using a microwave, three different programs were tried. The acid microwave-assisted digestion was used before for BMAA extraction from cyanobacteria (Baptista et al., 2011). As far as we know, this method was not yet used for BMAA extraction from animals, in these case marine invertebrates. So, we optimized this technique to obtain higher recovery efficiency of BMAA.

Table 5 shows the comparison of the 1000 µg/L BMAA standard recoveries, after different microwave-assisted hydrolysis.

Table 5. *Comparison of the 1000 µg/L BMAA standard recoveries after different microwave-assisted hydrolysis, with different time, temperature and powers.*

Power (W)	**Temperature (ºC)**	**Time (min)**	**%BMAA recovery**
300+400+500	>50	5+5+5	42,72
300	>50	10	27,94
<1000	90	20	96,14

By comparing the BMAA standard recoveries, it can be seen that the program with 90 ºC, 20 min and power limited to under 1000 W presents the best recovery, with 96,1%. The recovery percentages obtained with the BMAA standard shows that this extraction technique is suitable.

With these results, it was decided to compare the calibration curve of hydrolyzed standards of BMAA with the calibration curve of non-hydrolyzed aqueous standards and see if there was any difference between them. The figure 6 shows that comparison.

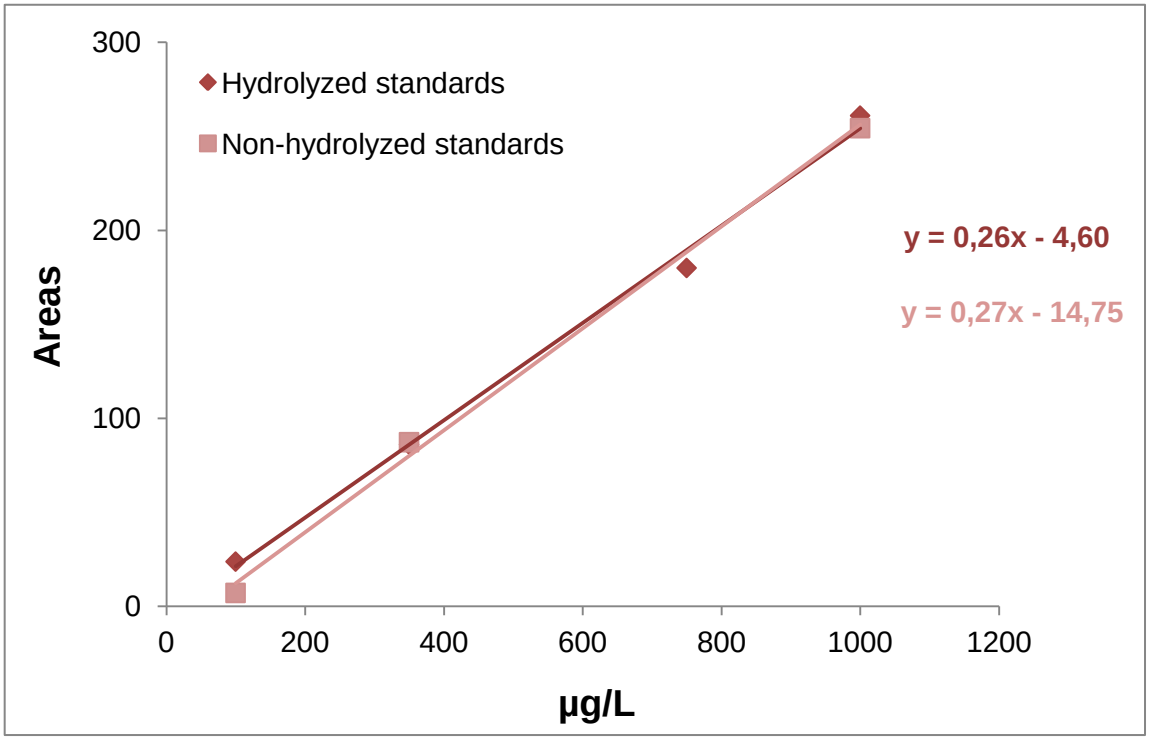

Figure 6. *Calibration curve of BMAA standards hydrolyzed at 90 ºC during 20 min and non-hydrolyzed aqueous standards. The trend line colored with dark pink correspond to the hydrolyzed standards and the trend line colored with light pink corresponds to non-hydrolyzed standards.*

It can be seen that both exhibited the same calibration curve. The slopes were similar, meaning that there are practically no losses of BMAA during the hydrolysis. A t-test was made and no significant differences were observed.

Mussel samples spiked with BMAA standards were digested in the same way. The results obtained were compared with the BMAA hydrolyzed standards. The slopes obtained were statistically different ($p \leq 0,05$) and for that reason the quantification of BMAA in mussel samples was made by using the calibration curve of mussels spiked with BMAA standard (figure 7).

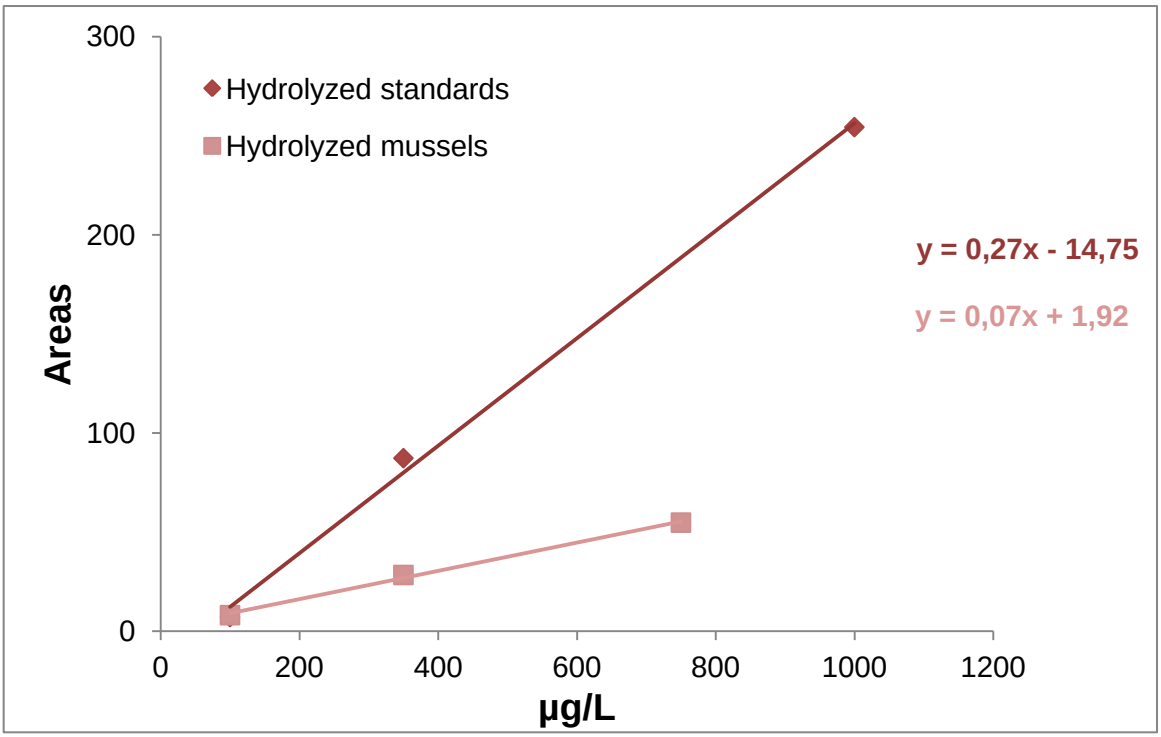

Figure 7. *Calibration curves of hydrolyzed standards and hydrolyzed mussels. The trend line colored with dark pink correspond to the hydrolyzed standards and the trend line colored with light pink corresponds to hydrolyzed mussels.*

The same was made with *G. umbilicalis*, where it was compared the calibration curve of snails spiked with BMAA standard and the calibration curve of BMAA hydrolyzed standards (figure 8).

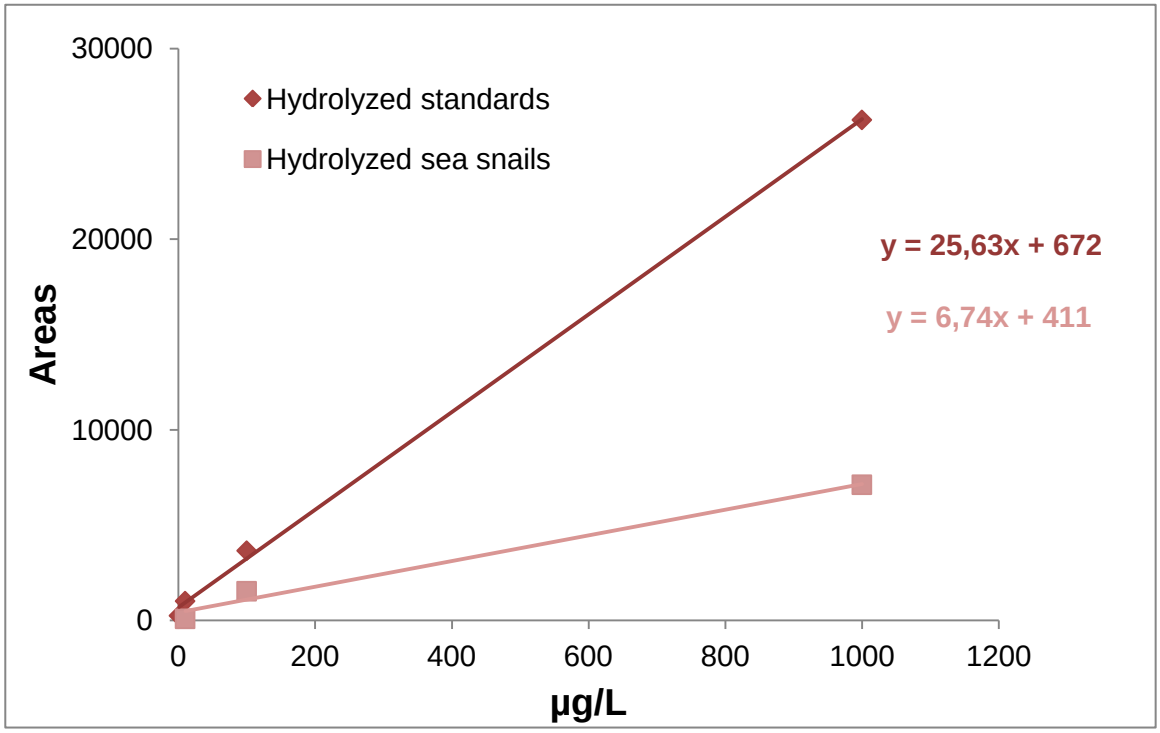

Figure 8. *Calibration curves of hydrolyzed standards and hydrolyzed sea snails. The trend line colored with dark pink correspond to the hydrolyzed standards and the trend line colored with light pink corresponds to hydrolyzed sea snails.*

From the figure it is possible to see that the two curves are different. The slopes are significantly different ($p \leq 0,05$), so, as it happened with mussels, the quantification of BMAA in sea snail samples was made by using sea snails spiked with BMAA standard.

3.2. Bioaccumulation of BMAA

3.2.1. Mussels exposed to BMAA standard

Mussels were able to bioaccumulate BMAA when they were exposed to three different concentrations of BMAA standard (1 mg/L; 2,5 mg/L and 5 mg/L) for 24 h and 48 h (figure 9).

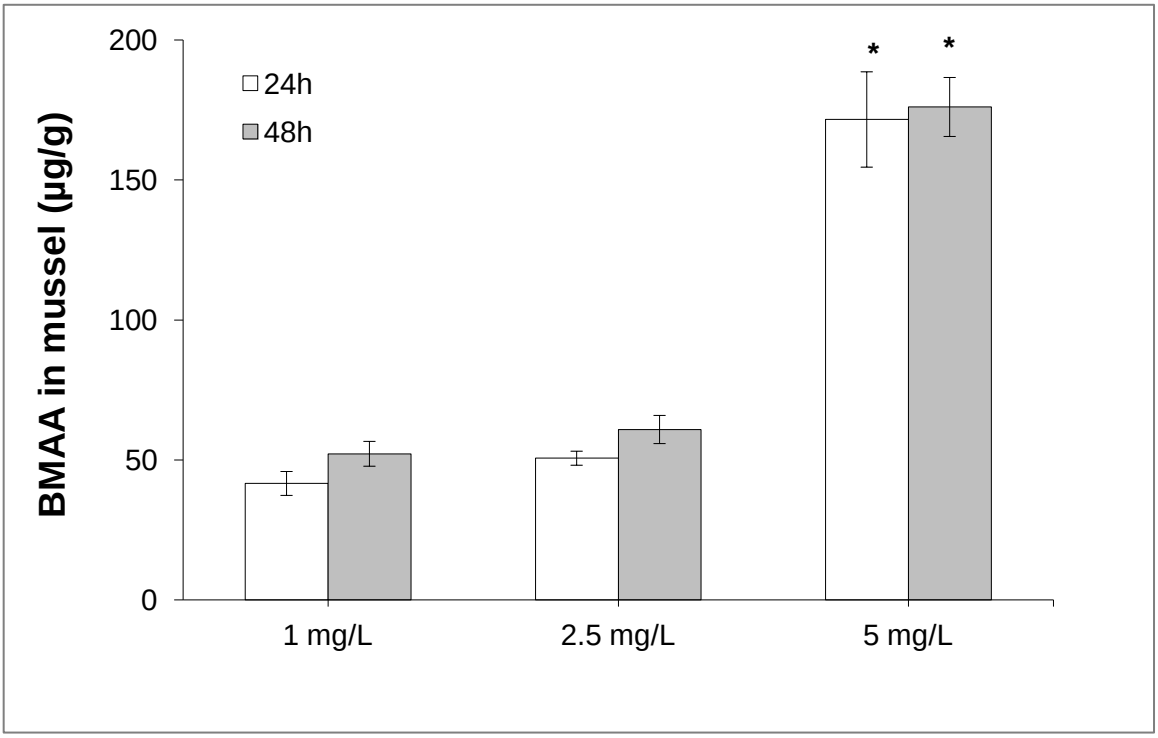

Figure 9. *BMAA levels (µg/g) in mussels exposed for 24 h and 48 h to different concentrations of BMAA (1 mg/L; 2,5 mg/L and 5 mg/L). Bars are the standard error (SE) of 4 replicates. * significantly different (p ≤ 0,05).*

As expected, there was an increase of BMAA concentration over time and these levels were higher at 5 mg/L. There was a significant difference at 5 mg/L, in both 24 h and 48 h, in comparison with the other two concentrations. However, no significant differences were observed between 1 mg/L and 2,5 mg/L.

Table 6 presents the concentration of BMAA on mussels exposed to 1 mg/L; 2,5 mg/L and 5 mg/L for 24 h and 48 h, and also the uptake rate for each day. It is possible to see that the uptake rate is higher in the first 24 h for any concentration used, and that the uptake rate decreased during the second 24 h.

Table 6. *Concentration of BMAA (µg/g) on mussels exposed to 1 mg/L; 2,5 mg/L and 5 mg/L for 24 h and 48 h and the respective uptake rate. Mean ± standard error (SE) for the concentration of BMAA.*

	BMAA µg/g (24 h)	**Uptake rate µg/g/h (24 h)**	**BMAA µg/g (48 h)**	**Uptake rate µg/g/h (48 h)**
1 mg/L	41,59 ± 4,27	1,73	52,19 ± 4,43	0,22
2,5 mg/L	50,62 ± 2,51	2,11	60,87 ± 5,03	0,21
5 mg/L	171,61 ± 17,01	7,15	176,08 ± 10,53	0,09

In figure 10 it is possible to see the deceleration of the uptake rates.

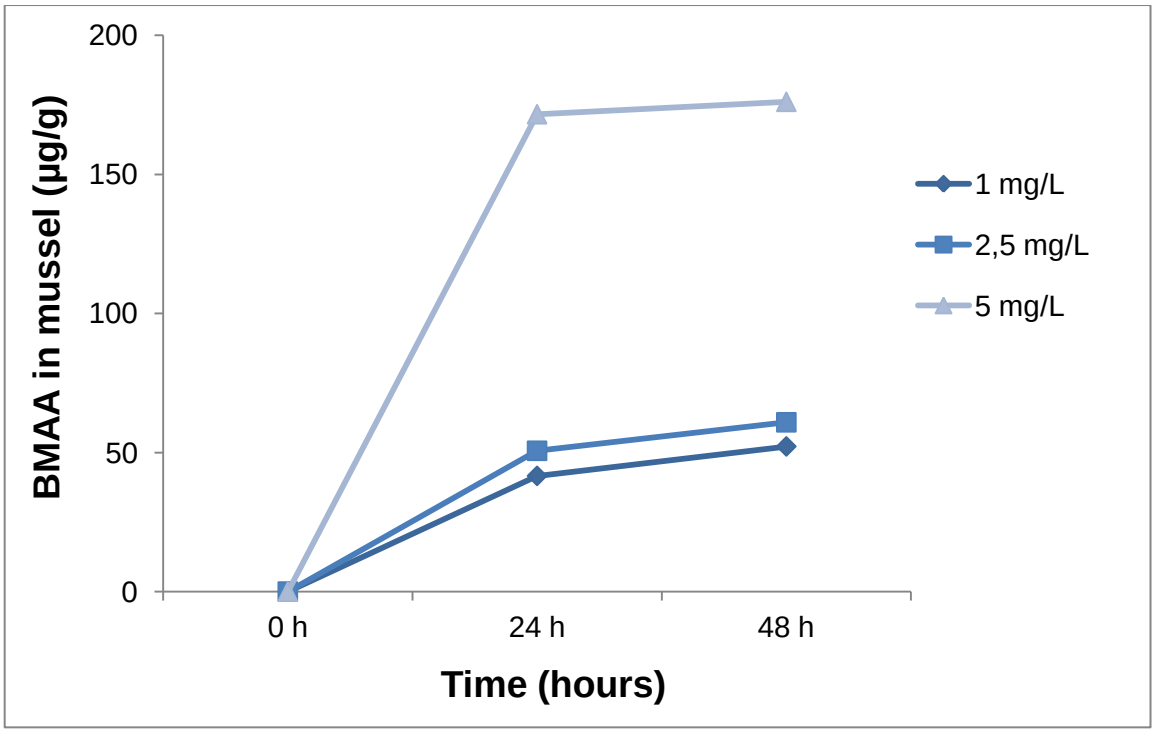

Figure 10. *Concentration of BMAA (µg/g) in mussels exposed to 1 mg/L, 2,5 mg/L and 5 mg/L during 24 h and 48 h.*

3.2.2. Animals fed with cyanobacteria

3.2.2.1. Cyanobacteria culture

Previous studies regarding BMAA production by cyanobacteria served as the basis for the choice of species (Cianca et al., 2012). To see how much BMAA would be incorporated by the species used for the bioaccumulation experiment, it was important to quantify the BMAA produced by the cyanobacteria strains used to feed these organisms. In case of mussels it was only possible to quantify the BMAA produced by each strain after the accumulation experiment.

Table 7 presents the quantification of BMAA in these strains. The strain of *Nostoc* sp. growth on BG_{11}^{15N} medium presented the highest concentration of BMAA. On the other hand, for *S. salina*, in both growth medium, the concentration of BMAA was lower than the limit of detection (LOD).

Table 7. *BMAA quantification (µg/g) in cyanobacteria strains used to feed the marine invertebrates. The different growth medium and organisms are also shown.*

Strain	**Medium**	**BMAA (µg/g)**	**Organism fed**
Nostoc sp. (LEGE#06077)	BG_{11}^{15N}	131,78	*G. umbilicalis*
Nostoc sp. (LEGE#06077)	MN	43,09	*G. umbilicalis*
Synechocystis salina (LEGE#06079)	BG_{11}^{15N}	<LOD	*G. umbilicalis*
Synechocystis salina (LEGE#06079)	Z8	<LOD	*M. galloprovincilais*

3.2.2.2. Mussels fed with cyanobacteria

Figure 11 presents the levels of BMAA in mussels digested with HCl and fed with cyanobacteria during 4, 8 and 10 days and 10 days of depuration. Although the concentration of BMAA was similar between 4 days and 8 days, it can be seen an increase of that level after 10 days. During the depuration period, there was a decrease of concentration of BMAA to 15,29 ± 0,66 µg/g . In terms of concentrations of BMAA, no statistically significant differences were observed between the different days of feeding ($p \leq 0,05$). However, a significant difference was observed at the depuration period.

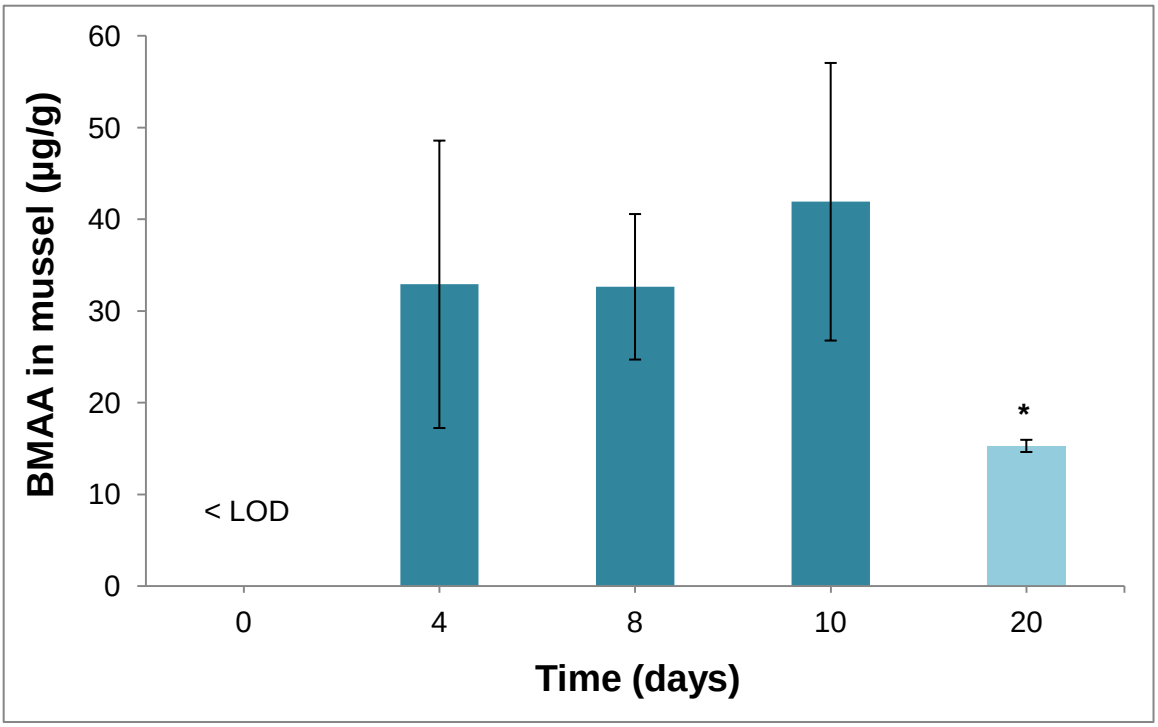

Figure 11. *BMAA quantification (µg/g) in mussel samples exposed to BMAA 4, 8 and 10 days of feeding and 10 days of depuration and acid-digested. The columns colored with dark blue corresponds to the feeding period and the column with light blue correspond to the depuration period. Bars are the standard error (SE) of 3 replicates. * significantly different ($p \leq 0,05$).*

Figure 12 shows the uptake rate of BMAA by the mussels during the bioaccumulation experiment. This rate was calculated using the concentrations obtained with the samples acid-digested. It is possible to see that the rate was maximum in the first 4 days of feeding, followed by a decrease of the rate during the rest of the feeding period. During the depuration period, and as expected, there was no ingestion, because no cyanobacteria were given to the mussels.

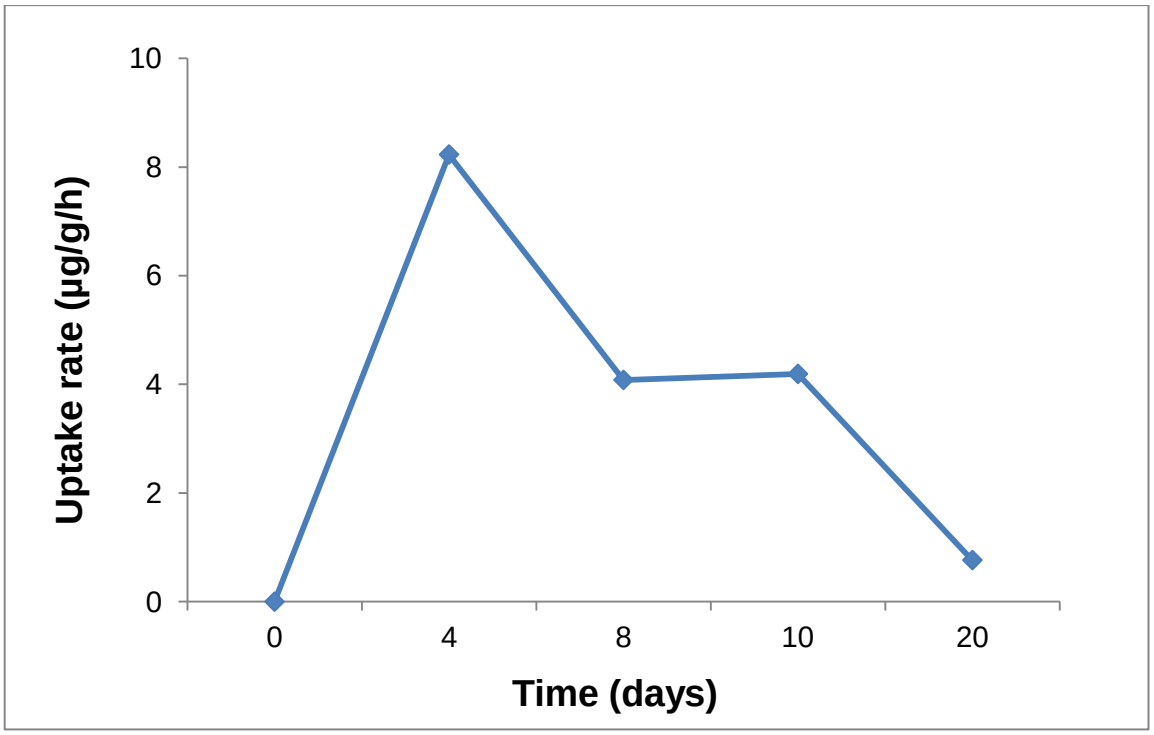

Figure 12. *Uptake rate of BMAA (µg/g/h) by mussels (acid-digested) during the bioaccumulation experiment.*

Figure 13 presents the BMAA quantification in mussel samples digested with pepsin.

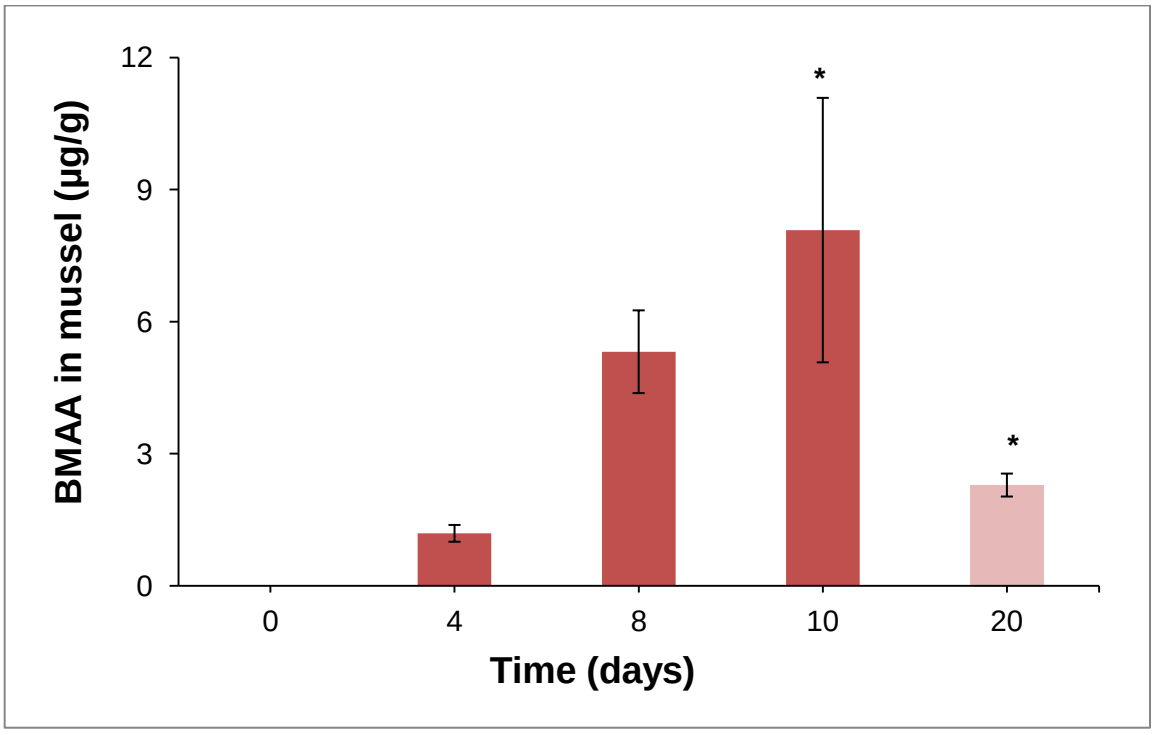

Figure 13. *BMAA quantification (µg/g) in mussel samples exposed to BMAA for 4, 8 and 10 days of feeding and 10 days of depuration and enzimatically digested with pepsin. The columns colored with dark pink correspond to the feeding period and the column with light pink corresponds to the depuration period. Bars are the standard error (SE) of 3 replicates. * significantly different (p ≤ 0,05).*

Figure 13 shows an increase of the concentration of BMAA during the feeding period, of which the highest concentration obtained was after 10 days with 8,08 ± 3,00 µg/g. A decrease during the depuration period was observed (2,29 ± 0,26 µg/g). No statistically significant difference was observed between 4 and 8 days ($p \leq 0,05$). However, between 4 days and 10 days and between 10 days and 20 days (depuration period) was observed a significant difference ($p \leq 0,05$).

Figure 14 presents the BMAA quantification in mussel samples digested with trypsin/chymotrypsin.

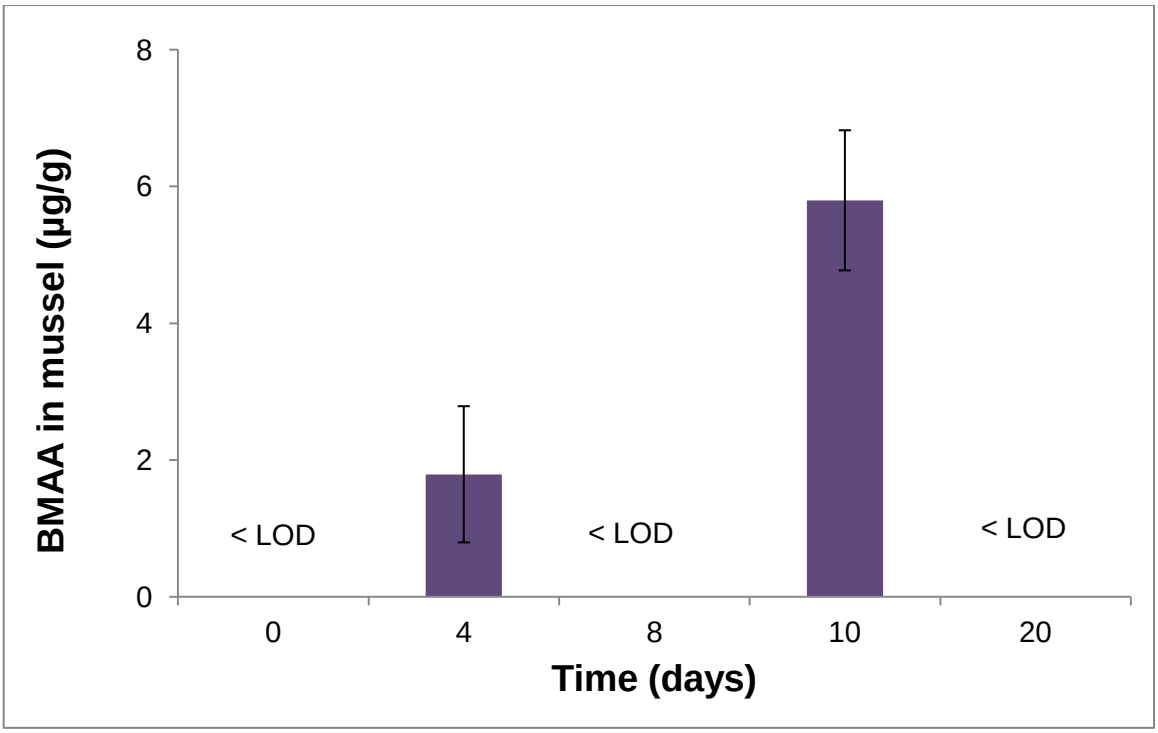

Figure 14. *BMAA quantification (µg/g) in mussel samples exposed to BMAA during 4, 8 and 10 days of feeding and 10 days of depuration and enzymatically digested with trypsin/chymotrypsin. The columns colored with dark purple correspond to the feeding period. Bars are the standard error (SE) of 3 replicates.*

From this figure, it can be seen that only at 4 days and 10 days were observed concentrations of BMAA. With 8 days and 20 days it was not possible to quantify the BMAA, since the concentrations were lower than de LOD. No significant differences were observed between 4 days and 10 days.

Table 8 shows the different concentrations of BMAA (µg/g) for the different days of exposure and for the different digestions used. It can be seen that the acid digestion was the one which removed more BMAA from the sample, followed by the pepsin digestion and the trypsin/chymotrypsin digestion.

Table 8: *BMAA quantification in mussels samples digested with HCl, pepsin and trypsin/chymotrypsin. Mean ± standard error (SE) (3 replicates).*

Samples	**HCl (µg/g)**	**Pepsin (µg/g)**	**Trypsin/chymotrypsin (µg/g)**
4 days	32,92 ± 15,66	1,19 ± 0,19	1,79 ± 0,99
8 days	32,65 ± 7,93	5,32 ± 0,94	< LOD
10 days	41,92 ± 15,13	8,08 ± 3,00	5,79 ± 1,02
20 days	15,29 ± 0,66	2,29 ± 0,26	< LOD

The results obtained with samples digested with pepsin were similar with those obtained with acid digestion, in which an increase of the level of BMAA could be seen during the feeding period followed by a decrease during the depuration period. However, the concentrations showed in these samples were lower than the ones obtained with the acid digestion. With the samples digested with trypsin/chymotrypsin it was only possible to see BMAA after 4 and 10 days of feeding. For 8 days and depuration period no BMAA was possible to quantify, since the values were lower than the limit of detection. However, it was also possible to see that the concentrations of BMAA obtained with these samples were lower than the ones obtained with the acid-digestion and the pepsin digestion.

All the results concerning BMAA quantification were obtained with the measurement of underivatized samples.

For the derivatized samples no results were obtained. This happened because, although in some samples it was possible to see the peak of BMAA, it was not possible to quantify since they were lower than the limit of detection. For example, for the lowest standard BMAA used (10 µg/L) the area obtained was 146,8, whereas for sample of 10 days of exposure it was of 38,9. However, it was possible to see that the area of the peaks followed the sample trend as presented here.

Mussels collect at the beach and mussels fed with *Chlorella sp*. as control did not present BMAA.

3.2.2.3. Sea snails fed with cyanobacteria

All the *G. umbilicalis* samples were derivatized. No results were obtained with *G. umbilicalis*, for both samples microwave-digested and enzymatically digested, as the samples were bellow the LOD.

3.3. Metabolic activity of cells of mussels fed with cyanobacteria

Table 9 presents the growth rate of cells isolated from the gills and hepatopancreas of *M. galloprovincialis* that was fed with cyanobacteria for 4, 8, and 10 days, following a 10-day depuration period.

Table 9. *Growth rate (h^{-1}) of cells from M. galloprovincialis after exposure to the Alamar Blue for 2,5 h, 5 h and 20 h. Mean ± standard deviation (SD) (3 replicates). * significantly different ($p \leq 0,05$).*

Gills	**2,5 h**	**5 h**	**20 h**
4 days	0,93 ± 0,03	1,35 ± 0,02	1,99 ± 0,02*
8 days	0,48 ± 0,03	0,79 ± 0,03	1,33 ± 0,05
10 days	0,60 ± 0,05	0,94 ± 0,07	1,51 ± 0,12
20 days	0,94 ± 0,02	1,14 ± 0,02	1,67 ± 0,06*
Hepatopancreas	**2,5 h**	**5 h**	**20 h**
4 days	1,14 ± 0,02	1,51 ± 0,02	2,04 ± 0,05*
8 days	0,57 ± 0,03	0,88 ± 0,03	1,70 ± 0,46
10 days	0,58 ± 0,01	0,87 ± 0,03	1,38 ± 0,06
20 days	0,65 ± 0,04	0,97 ± 0,03	1,57 ± 0,04

It is possible to see that the cells from the gills and hepatopancreas grew during the experiment. However, the growth rate was different for the different days of feeding. After 20 h of exposure to Alamar Blue, in cells from the gills it was observed that the growth rate of 4 days of feeding and 20 days (depuration period) was significantly higher than the

growth rate of 8 and 10 days of feeding. With the cells from hepatopancreas it was observed that the growth rate of 4 days was significantly higher than the rest of the feeding period and depuration period.

4. Discussion

4.1. Bioaccumulation experiment

In literature, it was reported the presence of BMAA in several marine animals, showing that biomagnification of BMAA may occur across trophic chain (Brand et al., 2010; Jonasson et al., 2010).

Various studies with cyanotoxins have shown that bivalves are able to accumulate toxins (Vasconcelos, 1995; Karlsson et al., 2003; Pereira et al., 2004; Pires et al., 2004; Saker et al., 2004 and Osswald et al., 2008). Among these reports there is a common agreement that bivalves are quite resistant to toxins. Feeding responses to toxic cells are widely used as good indicators of toxin accumulation and sensitivity in bivalves (Pereira et al., 2004),

In this work, accumulation of BMAA by mussels was shown. Mussels exposed to BMAA standard for 24 h and 48 h were able to uptake this amino acid, when provided with purposely higher concentrations. The results obtained showed that the mussels had higher uptake rate in the first 24 h and that rate decreased during the second 24 h of exposure.

Likewise, mussels fed with cyanobacteria (*S. salina*) showed bioaccumulation of BMAA. The increase in the levels of BMAA during the feeding period was not constant, since the amount that was uptake in the first 4 days was higher than was uptake after 8 and 10 days of feeding. After 10 days of depuration it was still possible to find levels of BMAA in the mussels, although these were significantly lower.

Similar accumulation results were obtained in previous studies dealing with cyanotoxins and mollusks. For example, Amorim and Vasconcelos (1999) reported accumulation of PST by *Anodonta cygnea* and Saker et al. (2004) reported accumulation of cylindrospermopsin by *A. cygnea*.

With anatoxin-a, a neurotoxin that has similar characteristics as BMAA (such as high level of solubility in water), no accumulation by *M. galloprovincialis* was observed (Osswald et al., 2008). Although this mussel was able to uptake anatoxin-a it did not accumulate it in very high rates and depurated it fast (Osswald et al., 2008). In this study it was observed an accumulation of BMAA. The fact that the mussel was able to accumulate

BMAA reinforces the idea that BMAA is predominantly bound to proteins, and not in the free form. This can help explain why BMAA, being soluble like anatoxin-a, can be accumulated by mussels.

It is noteworthy that mussels collected at the beach and mussels that were fed with *Chlorella sp*., as a control, did not present detectable BMAA. Therefore, the levels of BMAA present in the mussel fed with cyanobacteria were considered due to the feeding. Interestingly, the cyanobacteria used to feed the mussels did not present detectable levels of BMAA. These results show that the mussel was able to concentrate BMAA to a level high enough to be measured, thus supporting the BMAA bioaccumulation hypothesis, through the trophic chain.

The samples that were enzymatically digested showed similar results to the samples acid digested. As far as we know, enzymatic digestion has not yet been reported for BMAA extraction. This step was performed to simulate digestion processes in the stomach and small intestine of animals. In principle, *in vitro* digestion models provide a useful alternative to animal and human models by rapidly screening food ingredients (Hur et al., 2011). The enzymes used are known to be proteases and are present in the stomach (pepsin) and small intestine (trypsin/chymotrypsin). They are responsible for breaking down proteins/peptides into smaller peptides and amino acids (Hur et al., 2011).

A decrease in the levels of BMAA during the depuration period could be explained by detoxification of BMAA by the mussel, mediated by these digestive enzymes. Moreover, it can be hypothesized that similar BMAA releasing mechanisms will take place in organisms that feed on mussels. Since the enzymatic digestion allows the release of part of the total BMAA in the organism, it might also happen in the next trophic level. Amongst mussel predators are many marine organisms, such as fish or octopus, and also terrestrial organisms, such as humans.

Unlike what had happen for the mussel, none of the analyzed sea snail samples was positive for BMAA. This may have happened because this animal does not uptake or accumulates BMAA. Sea snails are grazers, and moving organisms, and might not have eaten the cyanobacteria provided in the bioaccumulation experiment or the cyanobacteria ingestion might not have been enough to quantify BMAA in the organism.

Another explanation can be the analytical method used. In this study two different methods were used, one using derivatization and another not using this step. The samples that were derivatized never allowed the quantification of BMAA.

Many discrepancies in published results on BMAA concentration can be seen in the literature (Banack et al., 2010a; Faassen et al., 2012; Glover et al., 2012). This may have happened because there is not a standard procedure to determine BMAA in various samples (e.g. cyanobacteria, marine invertebrates and vertebrates, etc.). Faassen et al. (2012) suggested that the differences in results may to be related to the analytical method used. It has been suggested that LC-MS/MS is a more selective and appropriate method for BMAA detection (Banack et al., 2010a; Faassen et al., 2012). Underivatized LC-MS/MS analysis and derivatized LC-MS/MS analysis are the most common methods used. The derivatization step is used to react with primary and secondary amino groups and make the molecule easier to detect and distinguish from structurally similar compounds by increasing its molecular weight (Banack et al., 2010a). However, some authors defend that this derivatization step is not specific, i.e. the derivatizing agent can react with all amino acids and other amino group containing compounds (Faassen et al., 2012). So there is always a chance that a derivatized compound other than BMAA has a similar retention time as the BMAA derivative (Faassen et al., 2012). In this study mussel samples were analyzed with both derivatization and without derivatization and sea snails samples were analyzed only with derivatization. Although the equipment used to analyze the derivatized samples is more sensitive than the one used to analyze the underivatized samples, no results were obtained with derivatized samples.

It has been also suggested that matrix effects may be complicating factors in BMAA analysis (Glover et al., 2012). Since, BMAA is a highly reactive molecule, it has the potential to interact with other molecules during the analysis, which may interfere with the accurate quantification (Li et al., 2010; Glover et al., 2012). Once a complex matrix is present, more chemical interactions can occur interfering with the analysis (Li et al., 2010; Glover et al., 2012). The failure to detect BMAA cannot be considered an absence of the compound.

4.2. Metabolic activity of cells of mussels fed with cyanobacteria

In this study, the metabolic activity of cells from the mussels fed with cyanobacteria was reported. The cytotoxicity of BMAA in mussels has not yet been investigated. In this work, we tested the effect of this neurotoxin on mussel cells of the gills and hepatopancreas by using Alamar Blue.

Given their feeding behavior, bivalves can accumulate molecules that are potentially dangerous for human health, including biotoxins (Louzao et al., 2010). However the

interaction of these toxins with the cells from bivalves can be very different. For example, palytoxin dramatically decreases the percentage of mantle and hepatopancreas cells (Louzao et al., 2010). Some toxins, like okadaic acid and azaspiracid-1 do not influence the viability of mussels cells (Louzao et al., 2010).

As it was possible to see in this work, BMAA can be accumulated by mussels. For this reason, it is important to see if this neurotoxin can affect the cells of *M. galloprovincialis*. The results showed that in both groups of cells (gills and hepatopancreas) there was an increase of the inhibition of metabolic activity, as the feeding period increased. These results show that BMAA affects bivalve cells, even when no differences can be seen in the accumulation of this toxin, indicating that the harmlessness of BMAA for mussels might be only apparent. Such data could be important to evaluate the real ecological impact of the toxin and can contribute to clarify how toxic molecules such as BMAA can be in some bivalves without any apparent effects.

It was also possible to see that, although the uptake rate of BMAA by mussels has a peak in the first 4 days, followed by a decrease, the growth rate of the cells from the gills and hepatopancreas of mussels fed during 8 and 10 days was lower than the cells from 4 days of feeding. That difference was higher in cells from hepatopancreas. Furthermore, the growth rate during the depuration period was lower in cells from the hepatopancreas than the cells from the gills, meaning that the cells from hepatopancreas were more affected than the others. This was expected since the hepatopancreas is an organ of the digestive tract of mollusks that is responsible for the absorption of digested food.

However, an important factor that has to be considered is the fact that the diet of the mussels is not mainly constituted by cyanobacteria. In fact, the most common food that is given to mussels that produces a higher growth rates and survival in aquaculture is mainly diatoms and flagellates (Davenport et al., 2000; Knauer and Southgate, 2007). So, partly the results obtained with the inhibition of the metabolic rate can be due to some stress as a response to the diet given, based only in cyanobacteria.

5. Conclusion

BMAA is a neurotoxin produced by cyanobacteria found in aquatic and terrestrial ecosystems. The ubiquity of cyanobacteria, including BMAA-producing strains, and the link between this toxin and neurodegenerative diseases pose a threat to humans. Recent findings of BMAA in different aquatic organisms suggest the occurrence of bioaccumulation in trophic chains.

With the mussels exposed to BMAA standard it was observed an accumulation of this neurotoxin and the uptake rate was higher at the first 24 h of exposure. For *M. galloprovincialis* fed with BMAA-producing cyanobacteria, an accumulation of BMAA was observed. With the enzymatic digestion of the mussels it was possible to see that BMAA might be transferred to the next trophic level, because these enzymes allowed the release of some BMAA from the organism.

Regarding the experiment with *G. umbilicalis*, no BMAA accumulation was observed. Some reasons can be given for this result. One of them is the way that sea snails feed. Maybe, because they are grazers, not enough cyanobacteria was eaten and therefore not sufficient BMAA was accumulated to be detected. So, the way of feeding of organisms can influence the quantity of BMAA ingested by them.

Two different methods were used in this study for BMAA analysis. Some samples were derivatized and the others were analyzed without derivatization. The samples that were derivatized never allowed the quantification of BMAA. Even though the equipment used for derivatized samples had a limit of detection lower than the equipment used for underivatized samples, it was not possible to detect BMAA in these samples. There are disagreements about which analytical method is better for BMAA detection. One of the complicating factors for this detection can be the matrix effect of biological samples. So, it is important to have a method that provides unambiguous identification and quantification of BMAA, since this neurotoxin has a link with neurodegenerative diseases.

The metabolic activity of cells from the mussels fed with cyanobacteria showed that in both cells used (gills and hepatopancreas) there was an increase of the inhibition of metabolic activity as the feeding period increased. One of the reasons for these results can be the fact that BMAA can affect cells from mussels. Although accumulation was observed in mussels without any apparent harm, BMAA can perturb the normal function of the cells. Other possible explanation can be the diet given to these mussels, during the

experiment. Because cyanobacteria were used exclusively to feed these organisms, there may have been some stress response that affected the metabolic activity of the cells. Nevertheless, further investigation should be done to better understand the mechanism of action of BMAA in marine invertebrates.

This study highlights the fact that there is a possible bioaccumulation of BMAA by *M. galloprovincialis*, an organism important in the aquatic ecosystem and present in the diet of many organisms, including humans. The fact that digestive enzymes allow the release of BMAA from mussels can foreseen a similar process into the organism that feed on them, enabling biomagnification of this neurotoxin through the trophic chain.

Further investigation on bioaccumulation of BMAA in higher levels of the trophic chain is needed. Concerning BMAA analysis, more investigation should be done to develop a standard procedure to detect and quantify BMAA in various complex samples, so more confidence on the results can be obtained.

6. Bibliography

Ahmed S.A., Gogal Jr.R.M. and Walsh J.E. (1994). A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to (3H)thymidine incorporation assay. *J. Immunol. Methods, 170*: 211-224.

Amorim A. and Vasconcelos V. (1999). Dynamics of microcystins in the mussel *Mytilus galloprovincialis*. *Toxicon, 37*: 1041-1052.

Backer L.C., Carmichael W., Kirkpatrick B., Williams C., Irvin M., Zhou Y., Johson T.B., Nierenberg K., Hill V. R., Kieszak S. and Cheng Y.S. (2008). Recreational exposure to low concentrations of microcystins during an algal bloom in a small lake. *Mar. Drugs, 6*: 389–406.

Banack S.A. and Cox P.A. (2003). Distribution of the neurotoxic nonprotein amino acid BMAA in cycas micronesica. *Botan. J. Lin. Soc., 143*: 165-168.

Banack S.A., Murch S.J. and Cox P.A. (2006). Neurotoxic flying foxes as dietary items for the Chamorro people, Marinas Islands. *J. Ethnoph., 106*: 97-104.

Banack S.A., Johnson H.E., Cheng R. and Cox P.A. (2007). Production of the neurotoxin BMAA by a marine cyanobacterium. *Mar. Drugs, 5*: 180-196.

Banack S.A., Downing T.G., Spácil Z., Purdie E.L., Metcalf J.S., Downing S., Esterhuizen M., Codd G.A. and Cox P.A. (2010a). Distinguishing the cyanobacterial neurotoxin β-N-methylamino-L-alanine (BMAA) from its structural isomer 2,4-diaminobutyric acid (2,4-DAB). *Toxicon, 5:* 868-879.

Banack S.A., Caller T.A. and Stommel E.W. (2010b). The cyanobacteria derived toxin beta-N-methylamino-L-alanine and amyotrophic lateral sclerosis. *Toxinc, 2*: 2837-2850.

Banack S.A., Metcalf J.S., Jiang L., Craighead D., Ilag L.L. and Cox P.A. (2012). Cyanobacteria produce N-(2-aminoethyl)glycine, a backbone for peptide nucleic acids which may have been the first genetic molecules for life on earth. *Plos one, 7*: 11.

Baptista M.S., Cianca R.C.C., Lopes V.R., Almeida C.M.R. and Vasconcelos V.M. (2011). Determination of the non protein amino acid β-N-methylamino-L-alanine in estuarine cyanobacteria by capillary electrophoresis. *Toxicon, 58*: 410-414.

Bartram J., Carmichael W.W., Chorus I., Jones G. and Skulberg O.M. (1999). Introduction. In: Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management (Chorus I. and Bartram J. eds.): 12-24. E&FN Spon, London.

Bauer E., Williams B.A., Voigt C., Mosenthin R. and Verstegen M.W.A. (2003). Impact of mammalian enzyme pretreatment on the fermentability of carbohydrate-rich feedstuffs. *J. Sci. Food Agric., 83*: 207-214.

Bell E.A. (2009). The discovery of BMAA, and exemples of biomagnification and protein incorporation involving other non-protein amino acids. *Amyo. Lat. Scler., 2*: 21-25.

Bláha L., Babica P. and Marsalek B. (2009). Toxins produced in cyanobacterial water blooms – toxicity and risks. *Interd. Toxic., 2*: 36-41.

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

Brito Â., Ramos V., Seabra R., Santos A., Santos C. L., Lopo M., Ferreira S., Martins A., Mota R., Frazão B., Martins R., Vasconcelos V. and Tamagnini P. (2012). Culture-dependent characterization of cyanobacterial diversity in the intertidal zones of the Portuguese coast: a polyphasic study. *Syst. applied microbio.*, *35*: 110.

Ceccherelli V.U. and Rossi R. (1984). Settlement, growth and production of the mussel Mytilus galloprovincialis. *Mar. Ecol. Prog. Ser., 16*: 173-184.

Chiu A.S., Gehringer M.M., Welch J.H. and Neilan B.A. (2011). Does α-amino-β-methylaminopropionic acid (BMAA) play a role in neurodegeneration?. *Int. J. Environ. Res. Public Health, 8*: 3728-3746.

Cianca R.C.C, Baptista M.S., Silva L.P., Lopes V.R. and Vasconcelos V.M. (2012a). Reversed-phase HPLC/FD method for the quantitative analysis of the neurotoxin BMAA (β-N-methylamino-*L*-alanine) in cyanobacteria. *Toxicon, 59*: 379-384.

Cianca R.C.C., Baptista M.S., Lopes V.R.L. and Vasconcelos V.M. (2012b). The non-protein amino acid β-N-methylamino-L-alanine in Portuguese cyanobacterial isolates. *Amino acids, 42*: 2473-2479.

Cohen S.A. (2012). Analytical techniques for the detection of α-amino-β-methylaminopropionic acid. *Anal., 137*: 1991-2005.

Cox P.A., Banack S.A. and Murch S.J. (2003). Biomagnification of cyanobacterial neurotoxins and neurodegenerative disease among the Chamorro people of Guam. *PNAS, 100*: 13380-13383.

Cox P.A., Banack S.A., Murch S.J., Rasmussen U., Tien G., Bidigare R.R., Metcalf J.S., Morrison L.F., Codd G.A. and Bergman B. (2005). Diverse taxa of cyanobacteria produce β-N-methylamino-L-alanine, a neurotoxic amino acid. *PNAS, 102*: 5074-5078.

Davenport J., Smith R.J.J.W. and Packer M. (2000). Mussels *Mytilus edulis*: significant consumers and destroyers of mesozooplankton. *Mar. Ecol. Prog. Ser., 198*: 131-137.

Doble A. (1999). The role of excitotoxicity in neurodegenerative disease: implications for therapy. *Pharmacol. Ther., 81*: 163-221.

Eriksson J., Jonasson S., Papaefthimiou D., Rasmussen U. and Bergman B. (2009). Improving derivatization efficiency of BMAA utilizing AccQ-Tag in a complex cyanobacterial matrix. *Amino Acids, 36*: 43-48.

Esterhuizen M. and Downing T.G. (2008). β-N-methylamino-L-alanine (BMAA) in novel South African cyanobacterial isolates. *Ecotox. Environ. Safety, 71*: 309-313.

Esterhuizen M., Pflugmacher S. and Downing T.G. (2011). β-N-methylamino-L-alanine (BMAA) uptake by aquatic macrophyte *Ceratophyllum demersum*. *Ecotox. Environ. Safety, 74*: 74-77.

Faassen E.J., Gillissen F., Zweers H.A.J. and Lurling M. (2009). Determination of the neurotoxins BMAA (β-N-methylamino-L-alanine) and DAB (α-, γ-diaminobutyric acid) by LC-MSMS in Dutch urban waters with cyanobacterial blooms. *Amyo. Lat. Scler., 2*: 79-84.

Faassen E.J., Gillissen F. and Lurling M. (2012). A comparative study on three analytical methods for the determination of the neurotoxin BMAA in cyanobacteria. *Plos One, 7*: 1-8.

Faassen E.J., Beekman W. and Lurling M. (2013). Evaluation of a commercial enzyme linked immunosorbent assay (ELISA) for the determination of the neurotoxin BMAA in surface waters. *Plos One, 8*: 1-7.

Ferrão-Filho A.S. and Kozlowsky-Suzuki B. (2011). Cyanotoxins: Bioaccumulation and effects on aquatic animals. *Mar. Drugs, 9*: 2729-2772.

Fleming L.E., Kirkpatrick B., Backer L.C., Bean J.A., Wanner A., Reich A., Zaias J., Cheng Y.S., Pierce R., Naar J., Abraham W.M: and Baden D.G. (2007). Aerosolized red-tide toxins (brevetoxins) and asthma. *Chest,131*: 187–94.

Glover W.B., Liberto C.M., McNeil W.S., Banack S.A., Shipley P.R. and Murch S.J. (2012). Reactivity of β-methylamino-L-alanine in complex sample matrixes complicating detection and quantification by mass spectrometry. *Anal. Chem., 84*: 7946-7953.

Holtcamp W., 2012. The emerging science of BMAA - do cyanobacteria contribute to neurodegenerative disease?. *Environ. Health Persp., 12*: 110-116.

Hur S.J., Lim B.O., Decker E.A. and McClements D.J. (2011). *In vitro* human digestion models for food applications. *Food Chem., 125:* 1-12.

Jiang L., Benoit A., Borggraeve W.M., Spacil Z. and Ilag L.L. (2012). Selective LC-MS/MS method for the identification of BMAA from its isomers in biological samples. *Anal. Bioanal. Chem., 403*: 1719-1730.

Johnson H.E., King S.R., Banack S.A., Webster C., Callanaupa W.J. and Cox P.A. (2008). Cyanobacteria (Nostoc commune) used as a dietary item in the Peruvian highlands produce the neurotoxic amino acid BMAA. *J. Ethnopharmacol, 118*: 159–165.

Jonasson S., Eriksson J., Lotta B., Spácil Z., Ilag L.L., Ronnevi L.O., Rasmussen U. and Bergman B. (2010). Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure. *PNAS, 107*: 9252-9257.

Karlsson K., Sipia V., Kankaanpaa H. and Meriluoto J. (2003). Mass spectrometric detection of nodularin and desmethylnodularin in mussels and flounders. *J. Chrom. B, 784:* 243-253.

Knauer J. and Southgate P.C. (1999). A review of the nutritional requirements of bivalves and the development of alternative and artificial diets for bivalve aquaculture. *Rev. Fish. Scien., 7:* 241-280.

Kotai J. (1972). Instructions for preparation of modified nutrient solution Z8 for algae. *Nor. Inst. Water Resea., 5.*

Kruger T., Monch B., Oppenhauser S. and Luckas B. (2010). LC–MS/MS determination of the isomeric neurotoxins BMAA (β-N-methylamino- L-alanine) and DAB (2,4

diaminobutyric acid) in cyanobacteria and seeds of Cycas revoluta and Lathyrus latifolius. *Toxicon, 55*: 547–557.

Kubo T., Kato N., Hosoya K. and Kaya K. (2008). Effective determination method for a cyanobacterial neurotoxin, β-N-methylamino-L-alanine. *Toxicon, 51*: 1264-1268.

Li A., Tia Z., Li J., Yu R., Banack S.A. and Wang Z. (2010). Detection of the neurotoxin BMAA within cyanobacteria isolated from freshwater in China. *Toxicon, 55*: 947–953.

Liu, X., Rush, T., Zapata, J. and Lobner, D (2009). [beta]-*N*-methylamino-L-alanine induces oxidative stress and glutamate release through action on system Xc−. *Exp. Neurol., 217*: 429-433.

Lobner D., Piana P.M.T., Salous A.K. and Peoples R.W. (2007). β-N-methylamino-L-alanine enhances neurotoxicity through multiple mechanisms. *Neurob. Dis., 25:* 360-366.

Lopes V., Fernández N., Martins R. and Vasconcelos V. (2010). Primary screening of the bioactivity of brackish water cyanobacteria: toxicity of crude extracts to *Artemia salina* Larvae and *Paracentrotus lividus* Embryos. *Mar. Drugs, 8*: 471-482.

Louzao M.C., Espiña B., Cagide E., Ares I.R., Alfonso A., Vieytes M.R. and Botana L.M. (2010). Cytotoxic effect of palytoxin on mussel. *Toxicon, 56*: 842-847.

Majoor-Krakauer D., Willems P.J. and Hofman A. (2003). Genetic epidemiology of amyotrophic lateral sclerosis. *Clin. Genet., 63*: 83-101.

Metcalf J.S., Banack S.A., Lindsay J., Morrison L.F., Cox P.A. and Codd G.A. (2008). Co-occurrence of β-N-methylamino-L-alanine, a neurotoxic amino acid with other cyanobacterial toxins in British waterbodies, 1990-2004. *Environ. Microbiol., 10*: 702-708.

Metcalf J.S., Banack S.A., Kotut K., Krienitz L. and Codd G.A. (2013). Amino acid neurotoxins in feathers of the Lesser Flamingo, *Phoeniconaias minor*. *Chem., 90*: 835-839.

Moura S., Ultramari M.A., Paula D.M.L., Yonamine M. and Pinto E. (2009). 1H NMR determination of β-N-methylamino-L-alanine (L-BMAA) in environmental and biological samples. *Toxicon, 53*: 578–583.

Murch S.J., Cox P.A., Banack S.A., Steele J.C. and Sacks O.W. (2004a). Occurrence of β-methylamino-L-alanine (BMAA) in ALS/PDC patients from Guam. *Acta Neurol. Scand., 110*: 267-269.

Murch S.J., Cox P.A. and Banack S.A. (2004b). A mechanism for slow release of biomagnified cyanobacterial neurotoxins and neurodegenerative disease in Guam. *PNAS,* 101: 12228-12231.

National Institute of Environmental Health Sciences (NIEHS) (2008). Chemical information review document for *L*-β-methylaminoalanine (CAS No. 15920-93-1). *U.S Department of Health Sciences National Institutes of Health*: 1-40. (http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/LbetaMethylaminoalanine_508.pdf) accessed on February, 2013.

Osswald J., Rellán S., Gago A. and Vasconcelos V. (2008). Uptake and depuration of anatoxin-a by the mussel *Mytilus galloprovincialis* (Lamarck, 1819) under laboratory conditions. *Chem., 72*: 1235-1241.

Pablo J., Banack S.A., Cox P.A., Johnson T.E., Papapetropoulos S., Bradley W.G., Buck A. and Mash D.C. (2009). Cyanobacterial neurotoxin BMAA in ALS and Alzheimer's disease. *Acta Neurol. Scand., 120*: 216-225.

Paerl H.W. and Huisman J. (2008). Blooms like it hot. *Scien., 320*: 57.

Paul V.J. (2008). Global warming and cyanobacterial harmful algal blooms in Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs, H.K. Hudnell, Editor. 2008, U.S. Environmental Protection Agency: Springer US.: 239-257.

Pereira P., Dias E., Franca S., Pereira E., Carolino M. and Vasconcelos V. (2004). Accumulation and depuration of cyanobacterial paralytic shellfish toxins by the freshwater mussel *Anodonta cygnea*. *Aqua. Tox.,, 68*: 339-350.

Pires L.M.D., Karlsson K.M., Meriluoto J.A.O., Kardinaal E., Visser P.M., Siewertsen K., Donk E.V. and Ibelings B.W. (2004). Assimilation and depuration of microcystin-LR by zebra mussel, Dreissena polymorpha. *Aqua. Tox.,, 69*: 385–396.

Rao S.D., Banack S.A., Cox P.A. and Weiss J.H. (2006). BMAA selectively injures motor neurons via AMPA/kainite receptor activation. *Exper. Neurol., 201*: 244-252.

Rippka R., Deruelles J., Waterbury J. B., Herdman M. and Stanier R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.*, *111*: 1-61.

Rosén J. and Hellenas K.E. (2008). Determination of the neurotoxin BMAA (β-N-methylamino-L-alanine) in cycad seed and cyanobacteria by LC-MS/MS (liquid chromatography tandem mass spectrometry. *Anal., 133*: 1785-1789.

Saker M.L., Metcalf J.S., Codd G.A. and Vasconcelos V.M. (2004). Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel *Anodonta cygnea*. *Toxicon, 43*: 185-194.

Shaw P.J. (2005). Molecular and cellular pathways of neurodegeneration in motor neurone disease. *J. Neurosurg. Psychiatry, 76*: 1046-1057.

Silva M., Azevedo J., Rodriguez P., Alfonso A., Botana L.M. and Vasconcelos V. (2012). New gastropod vectors and tetrodotoxin potential expansion in temperate waters of the Atlantic Ocean. *Mar. Drugs, 10*: 712-726.

Silva M., Barreiro A., Rodriguez P., Otero P., Azevedo J., Alfonso A., Botana L.M. and Vasconcelos V. (2013). New invertebrate vectors for PST, spirolides and okadaic acid in the North Atlantic. *Mar. Drugs, 11*: 1936-1960.

Sivonen K. and Jones G. (1999). Cyanobacterial toxins. In: Toxic cyanobacteria in water: A guide to public health significance, monitoring and management (Chorus I. and Bartram J., eds.): 55-124. E&FN Spon, London.

Spácil Z., Eriksson J., Jonasson S., Rasmussen U., Ilag L.L. and Bergman B. (2010). Analytical protocol for identification of BMAA and DAB in biological samples. *Anal., 135*: 127-132.

Stommel E.W., Field N.C. and Caller T.A. (2013). Aerosolization of cyanobacteria as a risk factor for amyotrophic lateral sclerosis. *Med. Hypot., 80*: 142-145.

Vasconcelos V.M. (1995). Uptake and depuration of the heptapeptide toxin microcystin-LR in *Mytilus galloprovincialis*. *Aqua. Tox., 32*: 227-237.

Vega A. and Bell E.A. (1967). α-Amino-β-methylaminopropionic acid, a new amino acid from seeds of *Cycas circinalis*. *Phytochem., 6*: 759-762.

Vega A., Bell E.A. and Nunn P.B. (1968). The preparation of *L*- and *D*-α-amino-β-methylaminopropionic acids and the identification of the compound isolated from *Cycas circinalis* as the *L*-isomer. *Phytochem., 7*: 1885-1887.

Waterbury J. B. and Stanier R. Y. (1978). Patterns of growth and development in *pleurocapsalean* cyanobacteria. *Microbiol. Rev.*, *42*: 2.