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A Survey of Aquatic Organisms for the Cyanotoxin Beta-Methylamino-L-Alanine (BMAA)

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By

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ABSTRACT

A SURVEY OF AQUATIC ORGANISMS FOR THE CYANOTOXIN BETA-METHYLAMINO-L-ALANINE (BMAA)

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Cyanobacteria blooms are increasing globally as a result of eutrophication. Many cyanobacteria are potentially harmful, not only because of the oxygen depleted zones created when they decay, but also because of the toxins they produce. β-Methylamino-L-alanine (BMAA), reportedly produced by many species of cyanobacteria, is a non-proteinogenic amino acid that has been linked to the development of neurodegenerative diseases (amyotrophic lateral sclerosis (ALS) and Alzheimer's) in humans. This study presents results from an assessment of BMAA in cyanobacteria and their grazers from the lower Chesapeake Bay and Lake Erie regions. BMAA was analyzed in samples prepared with the EZ:Faast^{TM} kit using gas chromatography-mass spectrometry (GC-MS) as well as LTQ Orbitrap liquid chromatography-mass spectrometry (LC-MS). Even though detection limits were as low as 0.28 ng and 5.24 pg for the GC-MS and LC-MS methods, respectively, BMAA was not found in any cyanobacteria samples analyzed as part of this study. Likewise, BMAA was not detected in any fish collected from Sandusky Bay and Grand Lake St. Marys, or clam, oyster, and crab samples from the lower Chesapeake Bay. BMAA concentrations in blue crab samples previously collected from the upper Chesapeake Bay and analyzed by another laboratory were verified (9.5μg g^{-1} dry weight) using the methodologies reported here. Although this study did not detect BMAA in many samples, this may be because BMAA production is highly variable in the environment.

Further sampling is needed, especially in localities with dense cyanobacteria blooms, to assess the human exposure risk to this putative toxin.

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INTRODUCTION

Cyanobacterial blooms have increased globally as a result of nutrient over-enrichment in coastal, estuarine, and freshwater ecosystems (Conley et al., 2009; O'Neil et al., 2012; Paerl et al., 2011). The same is true of algal blooms in general (Anderson et al., 2002). Blooms are termed harmful when they elicit negative ecosystem effects. For example, bacterial decomposition of algal biomass following blooms can result in oxygen depleted zones that can be deadly to aerobic organisms (Paerl & Otten, 2013). In addition, species produce secondary metabolites that are known toxins that directly affect the animals that eat them or humans (Table 1) (Carmichael, 1992; Falconer, 1999; Van Dolah, 2000; Dow & Swoboda, 2002; Merel et al., 2013). Many of these toxins, including domoic acid and brevetoxin, are highly monitored due to the potential for human health effects through consumption of contaminated aquatic organisms and direct contact with contaminated water or aerosolized toxins (Anderson et al., 2002). The Food and Drug Administration (FDA) has established concentrations for many toxins at which actions, such as public warnings and additional water quality monitoring, by state agencies are required or highly recommended. For example, when toxin concentrations at or exceeding 0.8 ppm and 20 ppm for brevetoxin and domoic acid, respectively, are detected the FDA recommends state agencies take actions, such as water quality monitoring and possibly closing fisheries, in order to protect human health (FDA, 1998).

Table 1.
Known algal toxins, their producers, and associated symptoms.

Toxin	Associated toxicity	Organisms	Symptoms
Brevetoxin	Neurotoxic Shellfish Poisoning (NSP)	*Karenia brevis* (Dinoflagellate)	skin irritation, vertigo, chills etc.
Okadaic acid	Diarrhetic shellfish poisoning (DSP)	*Dinophysis* spp. (Dinoflagellates)	diarrhea, nausea
Ciguatoxin	Ciguatera Fish Poisoning (CFP)	*Gambierdiscus toxicus* (Dinoflagellate)	numbness, vertigo, nausea
Saxitoxins	Paralytic Shellfish Poisoning (PSP)	*Alexandrium* spp. *Gymnodinuim* spp. (Dinoflagellates)	nausea, vomiting, vertigo etc.
Domoic acid	Amnesic Shellfish Poisoning (ASP)	*Pseudo-nitzschia spp.* (Diatoms)	vomiting, diarrhea, headache etc.
Anatoxin-a	Neurotoxin	*Anabaena* spp. (Cyanobacteria)	loss of coordination, convulsions, respiratory paralysis
Nodularin	Hepatotoxin	*Nodularia* spp. (Cyanobcateria)	nausea, headaches
Microcystins	Hepatotoxins	*Microcystis* spp. (and other Cyanobacteria)	jaundice, visual disturbances, nausea

1.1. Cyanotoxins

Cyanobacteria are ubiquitous in many terrestrial and aquatic environments (Conley et al., 2009; O'Neil et al., 2012; Paerl et al., 2011). Because their growth is generally favored at higher temperatures (above 25 °C), cyanobacteria blooms are projected to increase in the future as a result of global warming (O'Neil et al., 2012; Paerl & Otten, 2013). Like other algal taxa, cyanobacteria generally thrive in systems

with relatively high concentrations of nutrients (i.e. nitrogen and phosphorus) (Carmichael, 1992; Merel et al., 2013). In general, a combination of warm temperatures and appropriate nutrient concentrations are observed coinciding with a bloom. As with most algal blooms, decomposition of algal biomass resulting in oxygen depleted zones is a concern, but cyanobacteria are also known to produce a wide range of secondary metabolites that are known toxins (Van Dolah, 2000; Dow & Swoboda, 2002). Secondary metabolites are compounds that serve no known primary function (i.e. respiration or metabolism) (Carmichael, 1992). These toxic metabolites produced by cyanobacteria are termed cyanotoxins, and include groups of toxins such as microcystins, nodularins, cylindrospermopsins, and anatoxins (Table 1) (Carmichael, 1997; Li et al., 2001). Like other algal toxins, cyanotoxins are known to have hepatotoxic, neurotoxic, and/or dermatoxic (affects skin) effects on humans (Merel et al., 2013). Humans are potentially exposed to cyanotoxins through recreation, bathing in, or consuming the water containing these compounds. They may also be exposed to these compounds through ingestion of other aquatic organisms that incorporate the compounds into their muscle tissue.

Many cyanotoxins have been rigorously studies and classified, however, there other toxins which are not as well understood. β-Methylamino-L-alanine (BMAA), reportedly produced by 95% of cyanobacteria genera (Table 2) (Cox et al., 2005), is a non-proteinogenic amino acid, which is not naturally encoded into the genetic code and is neither a non-essential or essential amino acid (Cox et al., 2003). BMAA is considered a polar base and consists of a carboxyl group, amino group, and a methyl amino side chain (Fig. 1). Reported concentrations of BMAA associated with

cyanobacteria range from 0 - 6478 μg g^{-1} dry weight (Cox et al., 2005). BMAA has potential links with neurodegenerative diseases, and has been shown to be transferred through the aquatic food webs (Banack and Cox, 2003).

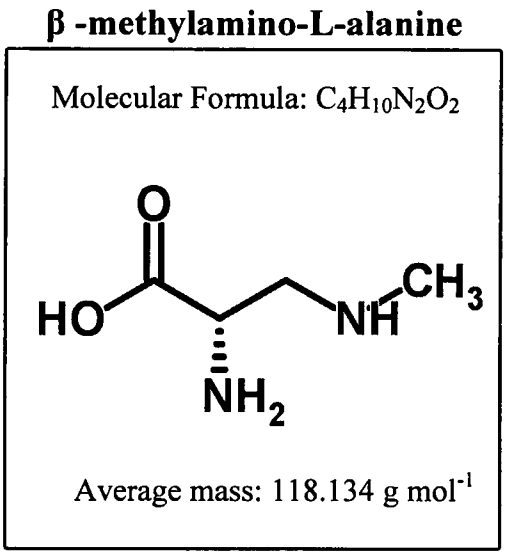

Fig 1. The chemical structures of β-methylamino-L-alanine (BMAA) redrawn from Esterhuizen-Londt *et al.* (2011).

Table 2.
Species of Cyanobacteria that are known to produce BMAA. Redrawn from Cox *et al.* (2005).

Cyanobacterial species/strain	Habitat	Origin
Microcystis PCC 7806	Freshwater	The Netherlands
Prochlorococcus marinus CCMP 1377	Marine	Sargasso Sea
Synechococcus PCC 6301	Freshwater	U.S.A.
Chroococcidiopsis indica GT-3-26	Marine rock	Unknown
Myxosarcina burmensis GB-9-4	Marine coral	Marshall Islands
Lyngbya majuscula	Marine	Zanzibar
Planktothrix agardhii NIES 595	Freshwater	Northern Ireland
Plectonema PCC 73110	Unknown	Unknown
Symploca PCC 8002	Marine, intertidal	U.K.
Trichodesmium thiebautii	Marine	Caribbean
Trichodesmium CCMP 1985	Marine, coastal	North Carolina
Anabaena variabilis ATCC 29413	Freshwater	U.S.A.
Aphanizomenon flos-aquae	Marine	Baltic Sea
Cylindrospermopsis raciborskii CR3	Freshwater	Australia
Nostoc PCC 7107	Freshwater	U.S.A.
Chlorogloeopsis PCC 6912	Soil	India
Fischerella PCC 7521	Hot spring	U.S.A.
Scytonema PCC 7110	Limestone cave	Bermuda

1.2. BMAA and neurodegenerative disease

In the mid-20th century, an unusually high number of cases (roughly 100 times more than reported anywhere else in the world) of a unique form of ALS, amyotrophic lateral sclerosis/Parkinsonism disease complex (ALS/PDC), were documented in the Chamorro tribe of Guam (Kurland and Mulder, 1954). It was suggested that an environmental factor might cause the high percentage of the disease, and that it may be

linked with the unique dietary practices of the tribe (Torres et al., 1957). In the 1960s, Arthur Bell hypothesized that β-N-oxalyl-amino-L-alanine (BOAA), a known neurotoxin, was prevalent in the diet of the Chamorro people (Vega & Bell, 1967). Instead of finding BOAA, Bell discovered BMAA associated with the Cycad tree (*Cycas micronesica*). Initially it was thought that BMAA was produced by the cycad tree, however it was many years later when scientists linked BMAA to the symbiotic cyanobacteria (*Nostoc*) associated with the roots of cycad trees (Cox et al., 2003). Although the toxicity of BMAA was not known, the hypothesis arose that the compound could potentially play a role in the high occurrence of ALS/PDC. Thus, the cycads, as well as the organisms that graze on them, were analyzed for BMAA content. It was originally hypothesized that the human route of exposure to BMAA was through the use of flour made from cycad seeds. It was shown later that the flour did not contain as much BMAA as the outer fleshy tissue of the cycad seed, and that ~90% of BMAA was removed from the flour during processing (Duncan et al., 1990; Banack & Cox, 2003). In search of alternative BMAA exposure routes, Cox and colleagues turned to the organisms that graze upon the cycad seeds. Flying foxes (*Pteropus mariannus*), another dietary staple of the Chamorro people known to consume cycad seeds, contained an average of 3,556 μg BMAA per gram of dried tissue (Cox et al., 2003; Banack & Cox, 2003). Therefore the hypothesis developed that BMAA was being produced by the symbiotic cyanobacteria associated with the cycads, then the flying foxes consumed the seeds containing BMAA, and the flying foxes were then consumed by the tribe members. In order to justify this brains of patients diagnosed with ALS/PDC and brains of those without the disease were analyzed. The brain samples of

the patients who died from the disease contained an average of 6.6 μg BMAA per gram of dried tissue (Cox et al., 2003); however, BMAA was not detected in patients who were not previously diagnosed with ALS/PDC.

Since this first link between BMAA and human exposure through the food web, there has been much controversy as to whether BMAA is linked with these fatal neurodegenerative diseases, and recent evidence suggests that it is. BMAA was not only found in brain tissue of ALS patients from the Chamorro tribe (Cox et al., 2003), but also in brain tissue of Alzheimer's patients in Canada (Murch et al., 2004) and ALS and AD patients from the University of Miami/National Parkinson Foundation Brain Endowment Bank (Pablo et al., 2009). BMAA was not found in brain tissue of patients that did not have these neurodegenerative diseases (Murch et al., 2004; Pablo et al., 2009). A recent study has shown a possible link between ALS and the consumption of blue crabs (*Callinectes sapidus*) in Annapolis, Maryland (Field et al., 2013). This study found BMAA incorporated into different organs of the blue crab, and maximum BMAA concentrations of 115.2 μg BMAA per gram of dried tissue. This study involved a small survey of three humans with ALS, and determined that seafood was a staple of their diet. Although the brains of those patients were not analyzed and the link is merely correlative, the potential exists that clusters of humans with unique diets may be exposed to BMAA and develop neurodegenerative diseases.

1.3. BMAA neurotoxic mechanisms

Amyotrophic lateral sclerosis (ALS), commonly known as Lou Gehrig's disease, and Alzheimer's disease (AD) are neurodegenerative diseases that kill large motor neurons associated with the brain and/or spinal cord (Rowland & Shneider,

2001). Like many diseases, ALS and AD can occur as either familial or sporadic cases. Familial cases only account for 5 to 10% of all cases (Mitchell & Borasio, 2007; Rowland & Shneider, 2001). Aside from random genetic mutations leading to disease development, another idea exists regarding the causation of sporadic cases. Scientists believe that glutamate may play a role in disease development (Vogels et al., 1999), given that ALS patients have been found to have increased levels of glutamate in the brain cells (Plaitakis & Caroscio, 1987). Cells of humans with early onset of ALS were analyzed and an increase in glutamate by nearly 100% compared to controls was observed (Plaitakis & Caroscio, 1987). Glutamate is the most abundant excitatory neurotransmitter in the brain, and levels of intracellular calcium ions (Ca^{2+}) are ultimately affected by the concentrations of extracellular glutamate (Vogels et al, 1999). Glutamate levels are correlated with the cell's intracellular calcium level, and it has been shown that increased levels of Ca^{2+} within a cell can trigger mitochondrial damage, reactive oxygen species, and potentially trigger cell apoptosis (Nunn, 1989; Weiss & Choi, 1988). Thus, it is thought that increased levels of glutamate might play a role in development of neurodegenerative diseases, specifically ALS.

In the decades following its discovery, the mode of toxicity of BMAA was debated. One theory suggests that BMAA, in the presence of bicarbonate, interferes with the functions (i.e. Ca^{2+} influx into the cell) of N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methly-4-isoxazolepropionic (AMPA) receptors (Nunn, 1989; Weiss & Choi, 1988; Weiss et al., 1989; Brownson et al., 2002). These receptors, which often occur at the same synapse, are activated by glutamate and studies have shown that hyperactivation of the receptors can lead to neuronal injury (Doble, 1999;

Rao et al., 2006; Lipton, 2004), mainly due to an increase in intracellular calcium (Rowland & Shneider, 2001). Experimental studies have shown that BMAA concentrations as low as 100 μM and 30 μM can activate NMDA and AMPA receptors, respectively (Rao et al., 2006; Lobner et al., 2009; Weiss & Choi, 1988). The increase in intracellular calcium due to BMAA binding to the glutamate receptors may result in a cascade of events that may lead to cell injury or death.

Another hypothesis suggests that BMAA mimics essential amino acids, including L-serine and glutamate (Rodgers & Shiozawa, 2008; Bell, E.A., 2009), and thus is mis-incorporated into proteins. When amino acids, including BMAA, are mis-incorporated, protein folding becomes irregular (Rodgers & Shiozawa, 2008). Many misfolded proteins are often disposed of by protein quality control (PQC) systems, but it has been shown that the PQC systems can be overwhelmed, either due to cell age or disease, leading to aggregation of the misfolded proteins (Gregersen et al., 2006). The accumulated proteins may be degraded or assembled into other toxic compounds, as demonstrated by Gregersen *et al.* (2006). Therefore, BMAA has the potential to cause cell damage because of its ability to cause protein misfolding through mimicking essential amino acids.

1.4. Cyanotoxins, including BMAA, in aquatic organisms

Many cyanobacteria, including freshwater, brackish and saltwater species, have now been found to produce cyanotoxins (Van Dolah, 2000; Merel et al., 2013), including BMAA (Cox et al., 2005), that have been quantified in organisms associated with higher trophic levels. Since cyanobacteria are the primary producers in many aquatic food webs, they serve as a food source for primary consumers, such as filter

feeders (i.e. clams and oysters). Cyanotoxins have been found incorporated into the tissues of primary consumers, which suggests transfer of the toxins through aquatic food webs. For example, microcystins have been quantified in crustaceans, bivalves, gastropods, and zooplankton with concentrations ranging from 0 to ~1000 $\mu g\ g^{-1}$ (Ferrão-Filho & Kozlowsky-Suzuki, 2011). In addition, BMAA has been identified in many organisms that consume cyanobacteria (Table 3). BMAA was found in brain and muscle tissue of mollusks and fish on Sweden's west coast where cyanobacteria are abundant (Jonasson et al., 2010) suggesting that this compound was transmitted through the aquatic food web. Blue crab (*Callinectes sapidus*) muscle tissue, oyster (*Pinctada margaritifera*) mantle tissue, and shrimp (*Panaeus duorarum*) muscle tissue from South Florida had BMAA concentrations that ranged from not detectable to as much as 7,000 $\mu g\ g^{-1}$ (BMAA/g tissue) (Brand et al., 2010). Further, BMAA was also detected in blue crab collected in the northern Chesapeake Bay and crab consumption was high in ALS patients from Annapolis, MD (Field et al., 2013). The potential for proliferation of cyanobacteria due to warmer temperatures, the ability for these fauna to produce BMAA, and the reported intake and incorporation of the compound into organisms of higher trophic levels may present a risk to human health.

Table 3.
BMAA in aquatic organisms.

Common name (Species)	BMAA μg g^{-1}	Reference
Blue crab (*Callinectes sapidus*)	2286	1
Oyster (*Pinctada margaritifera*)	275	1
Puffer fish (*Sphoeroides parvus)*	1907	1
Shrimp (*Panaeus duorarum*)	1156	1
White perch (Pomoxis annularis)	1806	1
Blue crab (*Callinectes sapidus*)	115.2	2
Smelt (*Osmerus eperlanus*)	0.023	3
Herring (*Clupea harengus*)	0.01	3
Common oyster (*Ostrea edulis*)	0.14	3

1=Brand *et al.* (2010)

2=Field *et al.* (2013)

3=Jonasson *et al.* (2010)

1.5 Limits on toxin production

Although cyanotoxins are produced by many species of cyanobacteria, not all blooms are linked with toxicity. Therefore, the presence alone of a bloom does not necessarily mean the toxin is being produced by the cyanobacteria associated with it. Blooms can be mono-specific, which means they contain only one species of cyanobacteria, but blooms often contain multiple species or different strains on species (Merel et al., 2013). It is known that non-toxic and toxic strains of cyanobacteria exist (Merel et al., 2013); therefore it is possible to have a bloom occurring, but the cyanobacteria strains affiliated with it are non-toxic. Aside from strain variability, toxin production may also be altered by the nutrients and temperatures within the body of water (Merel et al., 2013). For example, microcystin production by *Microcystis* is known to be affected by temperature and nutrients (nitrogen and phosphorus) (Sivonen et al., 1990; Vezie et al., 2002; Davis et al., 2009). The particular effects of temperature

on microcystin might also be strain specific, and that may explain why studies have shown increases of toxin production at higher temperatures and decreases in toxins at lower temperatures (Sivonen et al., 1990; Davis et al., 2009). However, there is a general consensus that increases in nitrogen and phosphorus concentrations increase the production of microcystins (Sivonen et al., 1990; Vezie et al., 2002). Aside from environmental factors, growth phase (lag, exponential, or stationary) of the cyanobacteria may play a role, as it is known that microcystin production is highest when the rate of cell division is greatest (Orr & Jones, 1998). Although the limitations of production of other cyanotoxins have been studied, the effects of these factors on BMAA production are not well understood. The only known study suggests that starving *Microcystis* of nitrogen can increase production of BMAA (Downing et al., 2010). No other studies have been able to determine which environmental or physiological factors limit the production of BMAA by cyanobacteria. The unknowns surrounding BMAA production limitations might explain why the concentrations reported in the aquatic organisms vary so greatly (Table 3).

1.6. BMAA detection

Although there are a variety of published methods that have shown that BMAA is present in cyanobacteria and their grazers, there is still an ongoing debate as to whether measurements of BMAA are accurate. A variety of analytical techniques have been used to quantify BMAA concentrations in natural samples, and these include: liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), ELISA kits, and ionophoresis (Banack et al., 2011). BMAA has an isomer, 2,4-diaminobutyric acid

(DAB), with a similar mass but different chemical structure (Fig. 1). It is crucial to differentiate between BMAA and its non-toxic isomer DAB (Spacil et al., 2010; Banack et al., 2011) to accurately quantify BMAA. Presence of the DAB isomer is thought to have caused overestimation of BMAA using analytical techniques that are unable to distinguish between the two compounds (Rosén & Hellenäs, 2008).

Aside from methodological differences, the natural degradation or flushing of BMAA from the tissue of aquatic organisms has not been taken into account in previous work. It is known that microcystins can be broken down through bacterial metabolism on the order of a day to a week or by photodegradation on a longer timescale (days to weeks) (Schmidt et al., 2014). Microcystins may also be flushed out of the tissues of aquatic organisms, specifically at a rate of 0.0828 day^{-1} for the snail (*Sinotaia histirca*) (Ozawa et al., 2003). The degradation and flushing rates, which have not been determined for BMAA, may also explain the variation in reported toxin concentrations.

1.7. Study Regions for the present study

The Chesapeake Bay and Lake Erie are home to extensive recreational and commercial fisheries. Both systems are adjacent to heavily urbanized areas and large human populations that come in contact with these waterways and the natural resources they support. Given that cyanobacteria are ubiquitous in many aquatic environments, and bloom conditions are often regulated by nutrient runoff, the Chesapeake Bay and Lake Erie are ideal study environments due to their proximity to urbanized areas with large human populations.

Typically, in the Chesapeake Bay estuary, cyanobacteria blooms occur in lower salinity waters during summer months when water temperatures are high and the water

column is stratified (Paerl & Huisman, 2008; Marshall et al., 2009). Many species of cyanobacteria (i.e. *Anabaena* spp., *Lyngbya* spp., *Planktothrix* spp.), and upwards of 126 taxa of cyanobacteria have been identified in the Chesapeake Bay estuary (Marshall et al., 2005), and likewise, toxins associated with these organisms have been identified. Microcystins have been detected and monitored in many areas of the Chesapeake Bay estuary (Tango & Butler, 2008; Bukaveckas et al., 2014). Between the years 2000 and 2006, microcystin levels were reported ranging from 2.9×10^{-2} to 6.58×10^{2} $\mu g\ L^{-1}$ (Tango & Butler 2008). The toxin levels reported exceeded the recommended safety level in drinking water for children (1 $\mu g\ L^{-1}$) in 71% of samples. Fish and bird kills have been associated with microcystin and anatoxin-a exposure, and beach closures have occurred in many years in the Chesapeake Bay region due to toxin levels surpassing safety guidelines (Tango & Butler, 2008). Also, BMAA was isolated in blue crab samples from the upper Chesapeake Bay at concentrations of 115.2 $\mu g\ g^{-1}$ (Field et al., 2013).

In Lake Erie, cyanobacteria blooms of *Microcystis*, *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis, Lyngbya, Nostoc* and *Planktothrix* have been reported (Cheung et al., 2013). *Microcystis* spp. and *Planktothrix* blooms occur regularly and often on an annual basis (Boyer, 2008; Saxton et al., 2011; Conroy et al., 2014), and it is thought that *Microcystis* can account for 42% of all cyanobacteria in Lake Erie (Rinta-Kanto et al., 2009). Toxic strains of *Microcystis* and *Planktothrix* have been reported and associated microcystin concentrations ranged from 1 to 400 $\mu g\ L^{-1}$ (Brittain et al., 2000; Murphey et al., 2003). Like the state of Virginia, Ohio has implemented safety

guidelines and plans of action during a toxic bloom. For example, microcystin concentrations exceeding 6 μg L^{-1} have been labeled as unsafe.

The Chesapeake Bay and Lake Erie regions have native cyanobacteria populations which are known to produce cyanotoxins. Few BMAA assessments have taken place in these regions, and given BMAA's reported toxicity and ability to be transferred through aquatic food webs, a survey of the Chesapeake Bay and Lake Erie cyanobacteria and fauna is warranted. In order to determine whether cyanobacteria and seafood from the lower Chesapeake Bay and Lake Erie regions (Sandusky Bay and Grand Lake St. Marys), cyanobacteria from natural water samples and fauna were collected in during periods with high and low cyanobacteria abundance and were analyzed as part of this study. Also, several cultures of cyanobacteria were analyzed with hopes of performing physiological experiments if BMAA was detected under natural conditions. The ultimate goals of this study were to determine if BMAA is being produced by cyanobacteria in the Chesapeake Bay and Lake Erie regions, and if BMAA is being incorporated into tissue of aquatic consumers in order to determine the potential for human exposure to this putative toxin.

CHAPTER 2

A SURVEY OF AQUATIC ORGANISMS FOR THE CYANOTOXIN B-METHYLAMINO-L-ALANINE (BMAA)

2.1. Introduction

Cyanobacteria blooms have increased as a result of eutrophication in estuarine and freshwater ecosystems (Conley et al., 2009; O'Neil et al., 2012; Paerl et al., 2011). Because their growth is generally favored at high temperatures, cyanobacteria blooms are projected to increase in the future as a result of global warming (O'Neil et al., 2012; Paerl & Otten, 2013). Decay of algal biomass following blooms can result in oxygen depleted zones that are deadly to aerobic organisms (Paerl & Otten, 2013). In addition, many harmful algal bloom (HAB) species produce secondary metabolites that are known toxins (Carmichael, 1992; Falconer, 1999; Van Dolah, 2000; Dow & Swoboda, 2002; Merel et al., 2013). In general, the toxins are differentiated based on the organism (i.e. diatom, dinoflagellate, and cyanobacteria) that produces them and their toxin type (i.e. hepatotoxin, neurotoxin). Many diatom and dinoflagellate species produce a variety of toxins, including domoic acid and brevetoxin, which are known neurotoxins (Van Dolah, 2000). Similarly, many species of cyanobacteria produce toxins, such as microcystin, saxitoxins, and anatoxins (Carmichael, 1992; Van Dolah, 2000), which are known to have harmful effects on animals and humans.

β-Methylamino-L-alanine (BMAA) (Fig. 1) is a naturally occurring non-proteinogenic, polar amino acid that has excitatory effects, which can often lead to a cascade of events (i.e. increases in intracellular calcium) that can cause nerve cell injury and possibly death (Choi, 1992), in its carbamate form (Rao et al., 2006; Lobner et al.,

2007). BMAA is produced by many species of cyanobacteria, including freshwater, brackish and saltwater species (Cox et al., 2005; Banack et al., 2007). It is thought to be transmitted to humans via the food web and has been found in brain tissue of amyotrophic lateral sclerosis (ALS) and Alzheimer's patients (Cox et al., 2003; Lobner et al., 2007; Field et al., 2013; Murch et al., 2004; Pablo et al., 2009). Given the devastating nature of these diseases, it is important to understand which localities contain BMAA producing bacteria and which organisms are incorporating BMAA in order to better understand the human exposure risk to this putative toxin.

Amyotrophic lateral sclerosis (ALS), commonly known as Lou Gehrig's disease, and Alzheimer's disease (AD) are neurodegenerative diseases that kill large motor neurons associated with the brain and/or spinal cord. Like many diseases, ALS and AD can occur as either familial or sporadic cases, however the former is attributed to only five to 10% of all cases (Mitchell & Borasio, 2007; Rowland & Shneider, 2001). The causes of the latter are not well understood, but many theories exist regarding the possibility of environmental factors playing a role in disease development (Rowland & Shneider, 2001). One compound recently linked with disease development is BMAA.

There are multiple hypotheses regarding the neurotoxic mechanism of BMAA, and its link to ALS and AD. Since BMAA is a glutamate receptor agonist, one theory suggests that BMAA, in the presence of bicarbonate, binds to N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptors (Nunn, 1989; Weiss & Choi, 1988; Weiss et al., 1989; Brownson et al., 2002) in the place of glutamate. NMDA and AMPA receptors are responsible for controlling the influx of calcium ions (Ca^{2+}) into nerve cells (Rowland & Shneider, 2001). These

receptors, which often occur at the same synapse, are activated by glutamate and studies have shown that hyperactivation of the receptors can lead to neuronal injury (Doble, 1999; Rao et al., 2006; Lipton, 2004), mainly due to an increase in intracellular calcium ions (Ca^{2+}) (Rowland & Shneider, 2001). It has also been shown that increases in intracellular Ca^{2+} can cause mitochondrial damage, reactive oxygen species (ROS) generation, and enzyme activation that can cause damage to the cell and potential lead to cell apoptosis {Esterhuizen and Downing, 2008). Studies have shown that BMAA concentrations as low as 100 μM and 30 μM have been found to activate NMDA and AMPA receptors, respectively (Rao et al., 2006; Lobner et al., 2009; Weiss & Choi, 1988).

Another theory suggests that BMAA mimics essential amino acids, including L-serine and glutamate (Rodgers & Shiozawa, 2008; Bell, E.A., 2009), and thus is mis-incorporated into proteins. When amino acids, including BMAA, are mis-incorporated, protein folding becomes irregular (Rodgers & Shiozawa, 2008). Many misfolded proteins are often disposed of by protein quality control (PQC) systems, but it has been shown that the PQC systems can be overwhelmed, either due to cell age or disease, leading to aggregation of the misfolded proteins (Gregersen et al., 2006). The accumulated proteins may be degraded or assembled into other toxic compounds, as demonstrated by Gregersen *et al.* (2006). Therefore, BMAA has the potential to cause cell damage because of its ability to cause protein misfolding through mimicking essential amino acids.

In the mid-20th century, an unusually high occurrence of amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS/PDC) was recorded among the

Chamorro people of Guam (Vega & Bell, 1967). Scientists suggested that there was an environmental toxin responsible for the increased occurrence of ALS/PDC (Vega & Bell, 1967). While searching for another proteinogenic amino acid, β-N-oxalyl-amino-L-alanine (BOAA), Arthur Bell identified BMAA in *Cycas micronesica* (Cycads) from Guam (Vega & Bell, 1967). At first, it was thought that BMAA was produced by the cycad tree, however, it was many years later when scientists linked BMAA to the symbiotic cyanobacteria (*Nostoc*) found associated with the roots of cycad trees (Cox et al., 2003). Initially, it was hypothesized that the human route of exposure to BMAA was through the use of flour made from cycad seeds. However, it was shown later that the flour, which is made from the inner layer of the seed, did not contain as much BMAA as the outer fleshy tissue of the cycad seed (Banack & Cox, 2003; Duncan et al., 2011). In search of alternative BMAA exposure routes, Cox and colleagues studied a cluster of ALS patients of the Chamorro people of Guam and were able to link the human consumption of flying foxes (*Pteropus mariannus*) with the disease in humans (Cox et al., 2003). Flying foxes, a staple of the Chamorro diet, ingest cycad seeds and BMAA was found to be biomagnified in their muscle tissue (Cox et al., 2003; Banack & Cox, 2003) and in the brain tissue of ALS patients (Cox et al., 2003).

Since this first link between BMAA and human exposure through the food web, there has been much controversy as to whether BMAA is linked with these fatal neurodegenerative diseases and whether BMAA has been accurately quantified in previous studies. Recent evidence suggests that BMAA is linked with disease. For example, BMAA was not only found in brain tissue of ALS patients from the Chamorro tribe (Cox et al., 2003), but also in brain tissue of ALS patients in Canada (Murch et al.,

2004) and ALS and AD patients from the University of Miami/National Parkinson Foundation Brain Endowment Bank (Pablo et al., 2009). BMAA, however, was not found in brain tissue of patients that did not have these neurodegenerative diseases (Murch et al., 2004; Pablo et al., 2009). Although the exposure history of ALS patients to dietary sources of BMAA is lacking in most cases, a recent study has shown a possible link between ALS and the consumption of blue crabs in Annapolis, Maryland (Field et al., 2013). Further studies are needed to strengthen the link between BMAA exposure through the food web and disease in human populations.

Many cyanobacteria, including freshwater, brackish and saltwater species, have now been found to produce BMAA (Cox et al., 2005). In addition, BMAA has been found in brain and muscle tissue of mollusks and fish on Sweden's west coast where cyanobacteria are abundant (Jonasson et al., 2010) suggesting that this compound is also transmitted through aquatic food webs. Blue crab (*Callinectes sapidus*) muscle tissue, oyster (*Pinctada margaritifera*) mantle tissue, and shrimp (*Panaeus duorarum*) muscle tissue from South Florida had BMAA concentrations that ranged from not detectable to as much as 7,000μg g^{-1} (BMAA/gram tissue) (Brand et al., 2010). Further, BMAA was also detected in blue crab collected in the northern Chesapeake Bay and crab consumption was high in ALS patients from Annapolis, MD (Field et al., 2013).

Although there are a variety of published methods that have shown that BMAA is present in cyanobacteria and their grazers, there is still an ongoing debate as to whether measurements of BMAA are accurate. A variety of analytical techniques have been used to quantify BMAA concentrations in natural samples, and these include: liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass

spectrometry (GC-MS), high performance liquid chromatography (HPLC), ELISA kits, and ionophoresis (Banack et al., 2011). BMAA has an isomer, 2,3-diaminobutyric acid (DAB), with a similar mass but different chemical structure (Fig. 1). It is crucial to differentiate between BMAA and its non-toxic isomer DAB (Spacil et al., 2010; Banack et al., 2011) to accurately quantify BMAA. Presence of the DAB isomer is thought to have caused overestimation of BMAA using analytical techniques that are unable to distinguish between the two compounds (Rosén & Hellenäs, 2008).

Chesapeake Bay and Lake Erie are home to extensive recreational and commercial fisheries. Both systems are adjacent to heavily urbanized areas and large human populations that come in contact with these waterways and the natural resources they support. Given that cyanobacteria are ubiquitous in many aquatic environments, and bloom conditions are often regulated by nutrient runoff, the Chesapeake Bay and Lake Erie are ideal study environments due to their proximity to urbanized areas with large human populations.

Typically, cyanobacteria blooms occur during summer months when water temperatures are high and the water column is stratified (Paerl & Huisman, 2008). In the Chesapeake Bay estuary, cyanobacteria blooms occur primarily in lower salinity waters during summer (Marshall et al., 2009). Microcystins, which are toxic compounds produced by species of *Microcystis*, have been detected and monitored in many areas of the Chesapeake Bay estuary (Tango & Butler, 2008; Bukaveckas et al., 2014). In Lake Erie, *Planktothrix* spp. and *Microcystis* spp. blooms are nearly an annual occurrence in Sandusky Bay, and many studies have found toxins associated with these species (Boyer, 2008; Saxton et al., 2011; Conroy et al., 2014). In order to

determine whether cyanobacteria and seafood from these two regions are contaminated with BMAA, I analyzed tissue from natural water samples and fauna collected in the lower Chesapeake Bay and Lake Erie regions (Sandusky Bay and Grand Lake St. Marys) during periods with high and low cyanobacteria abundance.

2.2. Methods

2.2.1. Clams, oysters, crab collection

Littleneck clams (*Mercenaria mercenaria)*, oysters (*Crassostrea virginica*), and blue crabs (*Callinectes sapidus)* were collected from a Lower Chesapeake Bay aquaculture facility (Cherrystone Aqua-farms, Cape Charles, VA) once a month between June and October 2014. Clams and oysters were donated by the facility and blue crabs were collected from a dock on the premises. Five organisms of each species were collected each month. Samples were kept on ice during transport, and once in the lab, organisms were immediately dissected (details below), tissue placed in 50mL falcon tubes, frozen at -80°C, lyophilized, and stored at ambient temperature until analysis (< 24 hours).

2.2.2. Fish/cyanobacteria collection

Representative species of fish were collected from the Lake Erie region, frozen, and shipped to ODU for dissection and analyses. Samples were collected on two dates at two different locations (Table 4). Cyanobacteria samples were also collected at the same sites. During the summers of 2013 and 2014 natural populations were dominated by *Planktothrix* spp. (pers obs Bullerjahn) and *Microcystis* spp. blooms (Conroy et al., 2014). Triplicate samples (1 L) were filtered onto GF/C filters (nominal pore size of 1.2 μm), frozen, lyophilized, and then shipped to ODU for analyses.

Table 4.
Sampling Areas. Location 1 (Sandusky Bay) and Date A (September 15, 2013). Location 2 (Grand Lake St. Marys) and Date B (July 7, 2014)

Species	Common Name	Location	Date
Lepomis macrochirus	Bluegill	1 & 2	A & B
Ictiobus cyprinellus	Buffalo fish	1	A
Carpiodes cyprinus	Quillback	1	A
Cyprinius carpio	Common Carp	1 & 2	A& B
Pomoxis annularis	Crappie	2	B
Ictalarus punctatuus	Channel Catfish	2	B
Dorosoma cepedianum	Gizzard Shad	2	B
Aplodinotus grunniens	Freshwater Drum	2	B

All soft tissues of the clams and oysters were homogenized before freeze drying. Backfin muscle, claw, and hepatopancreas of the crabs were dissected, stored, and analyzed separately because studies have shown varied β-methylamino-L-alanine (BMAA) concentrations in different crab tissues (Field et al., 2013). Dark and white muscle tissue was removed from each fish, homogenized, and stored at -80°C in 50 mL Falcon tubes until lyophilization.

All tissue samples were lyophilized for 48 hours using a LABCONCO FreeZone 6 Liter Benchtop Freeze Dry System (77520 Series). Freeze dried samples were then pulverized into a powder using a mortar and pestle.\

2.2.3. Cultures

Two isolates of *Anabaena* sp. (Florida and New Zealand), *Cylindrospermopsis raciborskii* (Florida), *Microcystis aeruginosa* (China), and *Lyngbya* sp. were donated from collections maintained at the University of North Carolina. *Anabaena* sp. were grown in CHU-10 medium in an environmental room maintained at 24°C. *Cylindrospermopsis raciborskii* was grown in Z80 medium and *Microcystis aeruginosa* and *Lyngbya* sp. were grown in BG-11 medium; all 3 isolates were grown in an environmental room maintained at 25°C. All cultures were kept under a 12 h light/12 h dark cycle with fluorescent lighting (6.65×10^{14} quanta cm^{-2} s^{-1}) in 1 L glass bottles, and were constantly stirred and aerated. Cyanobacteria samples were filtered onto GF/F filters, placed in cryovials and stored at -80°C. Prior to analysis, filters were transferred to 4mL amber glass vials and prepared for analysis as described below. A sterile GF/F was used as the sample blank to determine which compounds are introduced into the matrix from the filter itself.

2.2.4. Analytical Methods

Samples were prepared analyses using a Phenomenex EZ:Faast™ kit. This kit is specifically for amino acid analysis and includes a series of reagents used for liquid phase hydrolysis, solid phase extraction, and derivatization of hydrolysates (Table 5). Samples were first hydrolyzed using hydrochloric acid (6M) and heating (100°C). Hydrolysis was halted by adding Na_2CO_3 to samples until the pH was between 1.5 and 5 (tested with pH paper). The hydrolysates were then extracted using the solid phase EZ:Faast™ kit to remove unwanted compounds from the sample matrix. Amino acids retained on the EZ:Faast™ sorbent were then washed off of using the eluting medium

(Table 5). Finally, BMAA was derivatized to a non-polar compound with propyl chloroformate.

Table 5.
The reagents and their chemical makeup in the EZ:Faast™ kit.

Chemical Names	% of total mix	Common name
Norvaline Hydrochloric acid Water	0.0022 0.0365 99.9613	Ez:Faast™ Reagent 1: Internal Standard (Norvaline)
Sodium Carbonate Water	10.6 89.4	Ez:Faast™ Reagent 2: Sodium Carbonate Solution
Sodium Hydroxyde Water	1.32 98.68	Ez:Faast™ Reagent 3a: Eluting Medium 1
1-Propanol 3-Picoline	77 23	Ez:Faast™ Reagent 3b: Eluting Medium 2
Chloroform 2,2,4-Trimethylpentane Propyl Chloroformate	71.6 11 17.4	Ez:Faast™ Reagent 4: Organic Solution 1
2,2,4-Trimethylpentane Chloroform	81 19	Ez:Faast™ Reagent 5: Organic Solution 2
Iso-Octane Chloroform	80 20	Ez:Faast™ Reagent 6: Re-dissolution Solvent

GC-MS was used for quantification, identification, and separation of BMAA and 2, 4-diaminobutyric acid (DAB) (Esterhuizen and Downing 2008). Samples were analyzed on an Agilent GC 6890m Series System equipped with a Hewlett Packard 7683 Series Injector. The GC-MS was run under splitless mode with Helium carrier gas

through a J&W 122-5532 DB-5ms capillary column (30.0m x 250μm x 0.25μm). Data was analyzed with Agilent Chemstation software. BMAA standard (Sigma) and 2, 4-diaminobutyric acid (DAB) standard (Sigma) were analyzed in order to ensure the two isomers could be differentiated (i.e. no co-elution) using this method. Solid standard was weighed using an analytical balance and then treated in the same manner as environmental samples.

2.2.5. Detection limit

Because derivatized BMAA is not commercially available, a method detection limit was calculated using a BMAA standard that was processed in the same manner as environmental samples. A BMAA stock solution was made by weighing out 1 mg of BMAA standard and diluting that in 5 mL nanopure water (200 $\mu g\ mL^{-1}$). Triplicate standards (40 $\mu g\ mL^{-1}$) were made by diluting this primary stock solution. After accounting for dilution as a result of undergoing the hydrolyzation, neutralization, and derivatization steps described above, the final quantity of the BMAA standard injected into the GC-MS was 8.84 ng. Subsamples were taken from these standards and diluted with Reagent 6 (See Table 5) to create a dilution series (Table 6). BMAA detection limits were calculated using the slope of the regression lines and the standard deviation of the lowest detectable concentration for both the GC-MS and LC-MS (See next section) (Shrivastava & Gupta, 2011).

Table 6.
Dilution Series of BMAA standard

Dilution	Mass BMAA Injected (ng)
A	8.84
B	4.42
C	2.21
D	1.77
E	1.10
F	0.55

2.2.6. Verification of BMAA detection.

In addition to GC-MS analysis, derivatized (EzFaast™) samples were analyzed independently with a Thermo LTQ Orbitrap XL™ Hybrid Ion Trap-Orbitrap Mass Spectrometer following separation by an Agilent 1290 Infinity Binary HPLC. The HPLC was equipped with an Agilent ZORBAX Eclipse Plus C18 column (RRHD, 2.1 x 50 mm, 1.8 μm particles). The mobile phase consisted of 93:7 Water:Acetonitrile (Eluent A) and 0.1% Acetonitrile in water (Eluent B) using a flow rate of 200μL min^{-1} in the following gradient: 0.0 min= 100% A; 10.0 min= 80% A; 10.1 min= 0% A, 100% B; 13.0 min=0% A, 100% B; 15.0 min=100% A; 20.0 min= 100% A; 21.0 min= 100% A. The instrument was tuned with the propyl chloroformate derivative of the L-BMAA standard (Sigma), which resulted in similar mass and fragmentation patterns previously reported (BMAA 333 m/z ($M + H^+$)) (Banack et al., 2010; Downing et al., 2011). The Orbitrap detection limit was calculated in the same manner as described in for the GC-MS. However, the dilution series was altered due higher sensitivity of the Orbitrap.

2.2.7. Sample Biomass Analyzed

The amount of biomass analyzed varied slightly for each organism, however, an arbitrary minimum of 50 mg dry weight was hydrolyzed for each macroorganism (i.e. fish, oysters, crabs). This amount was determined based on the ability to detect and quantify BMAA in 5 mg of blue crab using our methods. For each lyophilized cyanobacteria, a minimum of 10 mg dry weight was analyzed. For *Planktothrix* samples, up to 30 mg dry weight was analyzed. The reason for different tissue weights was simply due to the amount of samples on hand. Given that the available cyanobacteria mass was much less than that of the macroorganisms, less cyanobacteria sample was used in order to insure enough was available for replicates. For those cyanobacteria that on GF/Fs, the samples were filtered until no more water would pass through the filter; this amount varied for each species depending on bloom/culture density.

2.2.8. BMAA quantification

In methodological trials, BMAA standard produced peaks with no co-eluting compounds. However, biological samples contained a co-eluting compound, n-propyl hexadecanoate ($C_{19}H_{38}O_2$). The extract ion chromatogram (EIC) function was used in the GC-MS full scan mode to differentiate BMAA from n-propyl hexadecanoate. The ions selected were m/z 130.1, which is the identifier ion of BMAA and m/z 61.0 which is the identifier ion of n-propyl hexadecanoate. Although the m/z 61.0 ion is found in BMAA, the EIC produces separate peaks for the two compounds based on retention times

2.3. Results

2.3.1. BMAA vs. DAB GC-MS

In order to determine that the GC-MS method could differentiate between BMAA and its isomer, DAB, derivatized (EZ:FaastTM) standards of both compounds were analyzed. BMAA and DAB derivatives had distinct retention times of 12.25 min and 13.55 min, respectively (Fig. 2). The mass spectra of the two compounds also differed, with an m/z 131 for BMAA and m/z 142 for DAB.

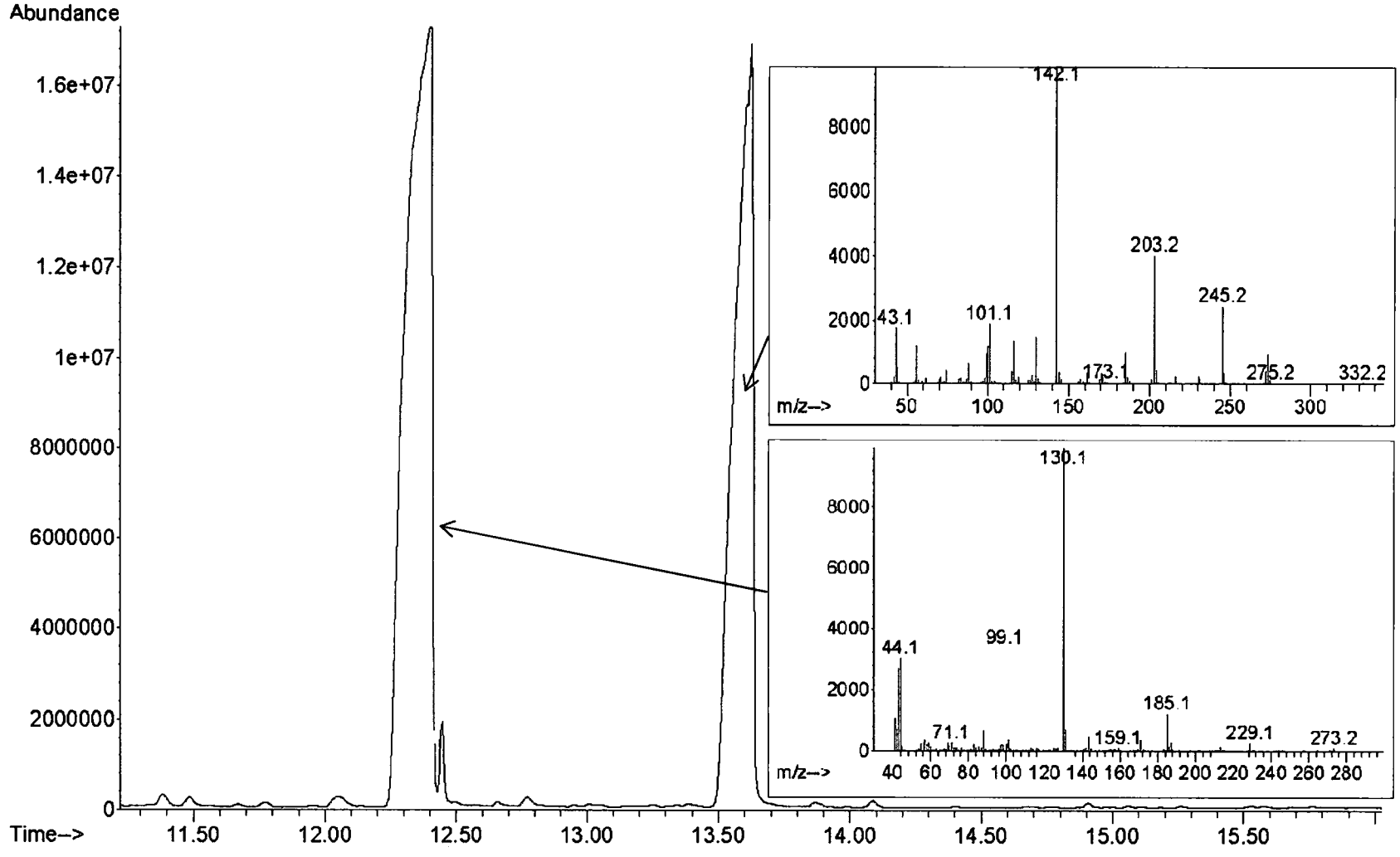

Fig. 2. GC-MS Total Ion Chromatogram (TIC) showing the distinct peaks of BMAA (Retention Time: 12.25 min; m/z : 131) and DAB (Retention Time: 13.55 min; m/z : 142)

A dilution series was used to determine the detection limit for BMAA (Fig. 3). Based on this standard curve ($R^2 = 0.99$), a detection limit of 0.28 ng was established. Derivatized BMAA standard was detectable, but not quantifiable, in injections containing 0.55 ng of BMAA, and was quantifiable in injection concentrations of 1.1 ng.

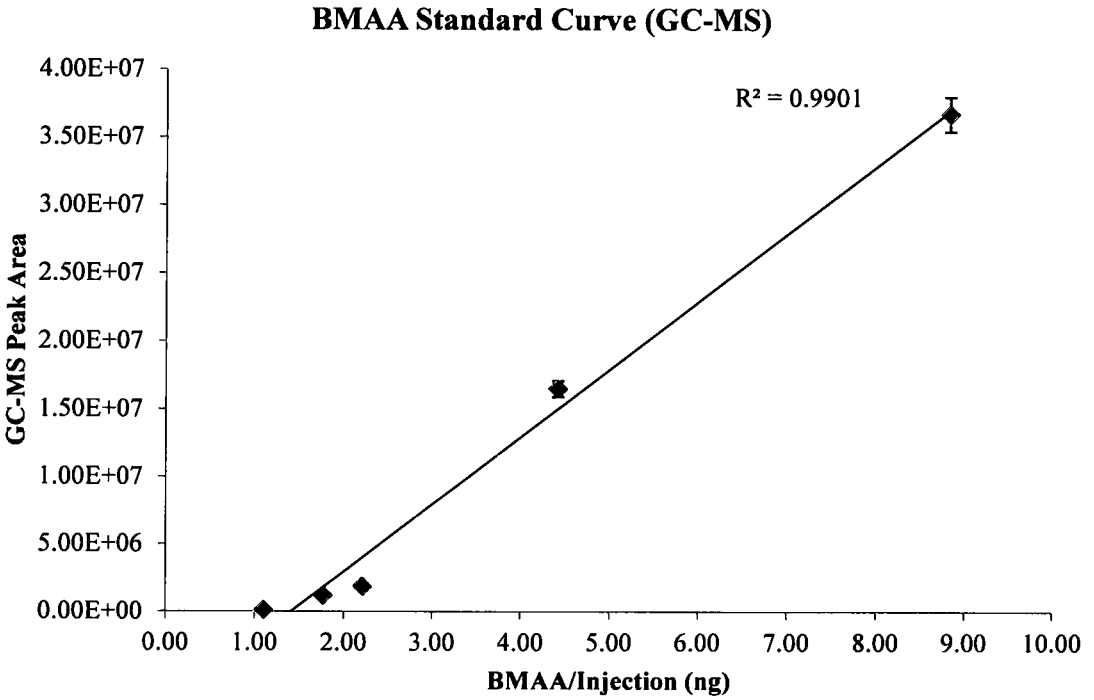

Fig. 3. Standard curve for L-BMAA (Sigma) using GC-MS ($R^2 = 0.99$). Error bars (standard deviations) may be covered by data symbol.

2.3.2. BMAA vs. DAB LC-MS Orbitrap

Using the LC-MS Orbitrap, derivatized BMAA and DAB standards had different retention times than those previously reported (Esterhuizen-Londt et al., 2011),

which may be attributed to the use of a different column; BMAA had a retention time of 11.49 min, and DAB a retention time of 11.55 min (Figs. 5 & 6, respectively). Although DAB eluted slightly later than BMAA, the compounds had very similar mass spectra and the identical M+ of 333.2. The product ions of the two compounds when fragmented were nearly identical to those previously reported (Figs. 5 & 6) (Esterhuizen-Londt et al., 2011). Both compounds had m/z peaks 273 and 187, but the product ion 245 was conspicuously absent in the fragmentation pattern for DAB.

The detection limit of BMAA for the LC-MS Orbitrap (5.24 pg) was 2 orders of magnitude lower than the GC-MS (Fig. 4). The deviation between replicates was much greater than for the GC-MS method but this may be because of the low concentrations.

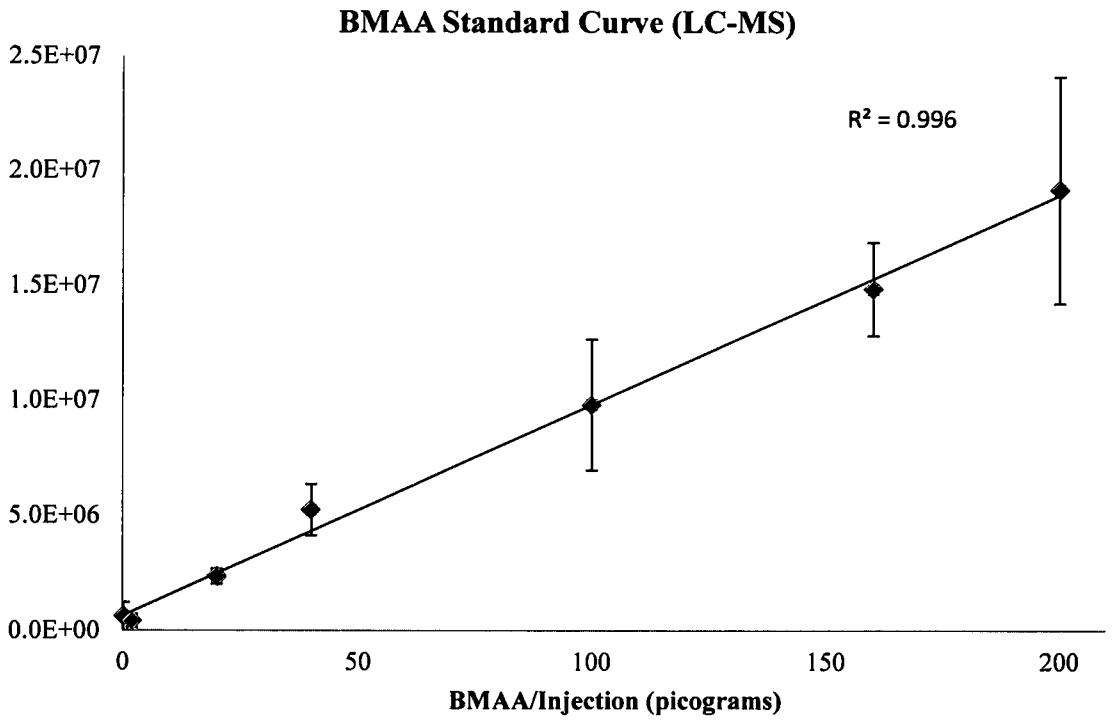

Fig. 4. Standard curve for L-BMAA (Sigma) using LC-MS Orbitrap ($R^2 = 0.996$).

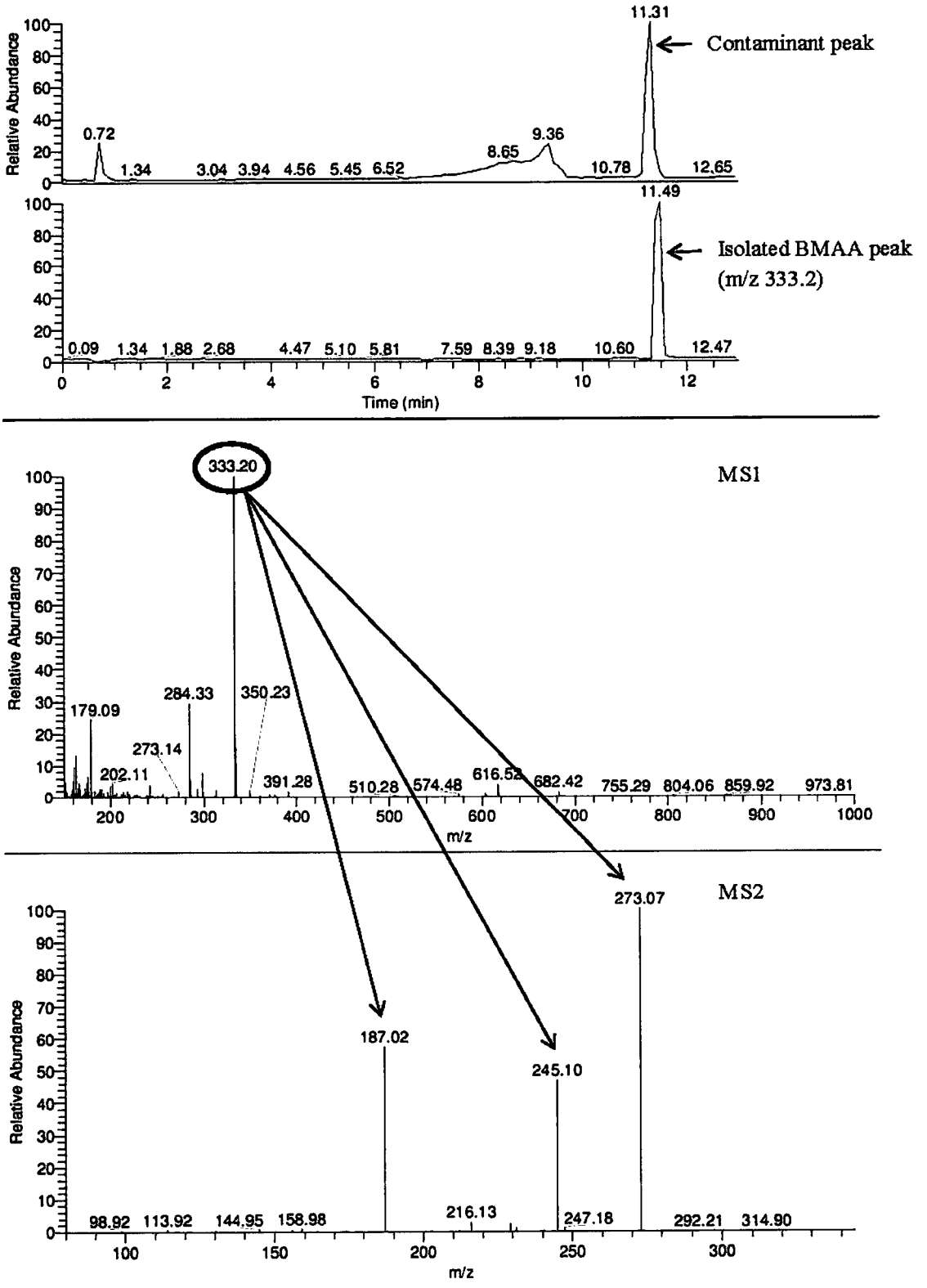

Fig. 5. Chromatogram and spectra of BMAA standard after analysis with LC-MS Orbitrap. MS2 represents the fragmentation of the m/z 333.2 ion.

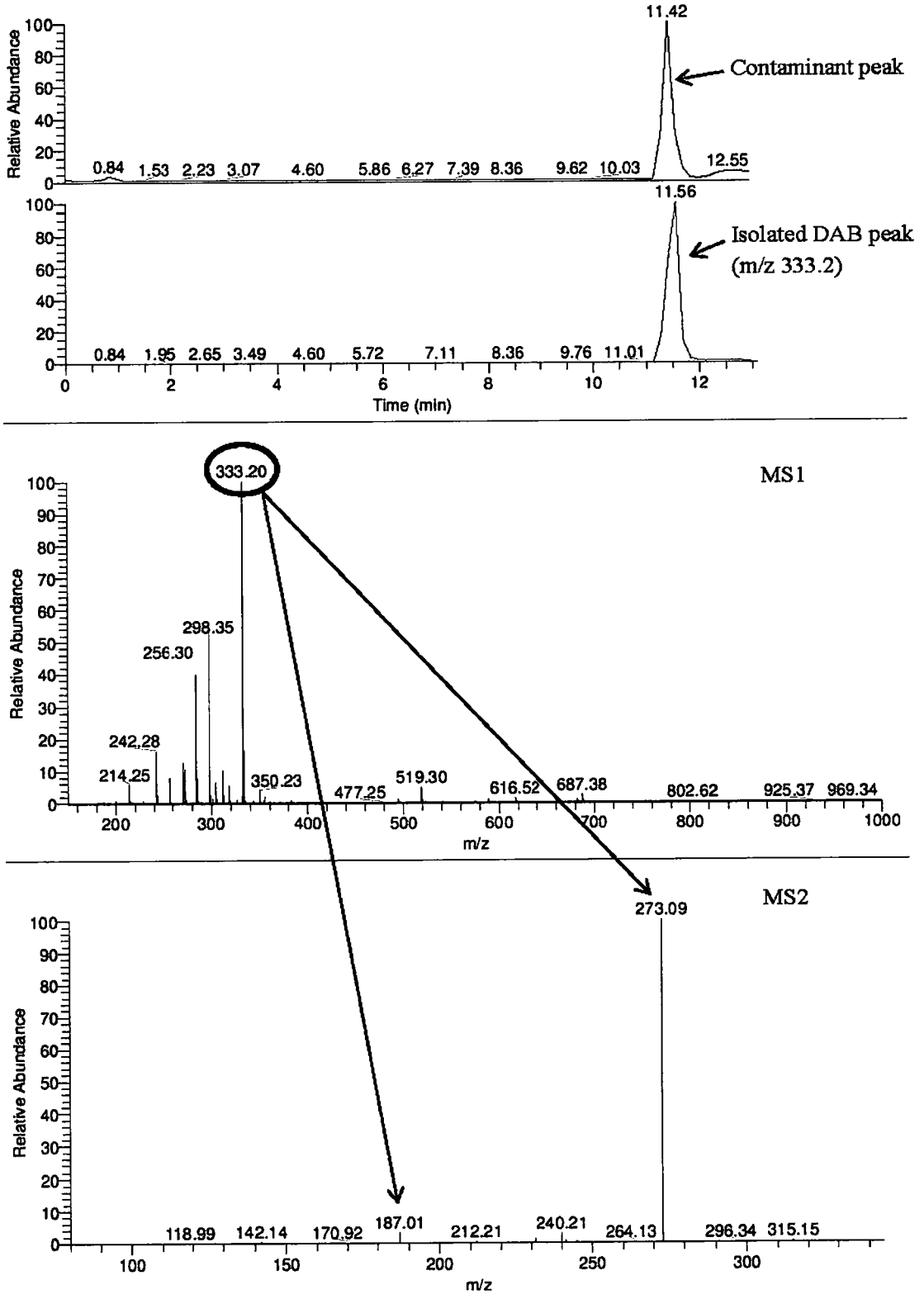

Fig. 6. Chromatogram and spectra of DAB standard after analysis with LC-MS Orbitrap. MS2 represents the fragmentation of the m/z 333.2 ion.

2.3.3. Biological Samples

None of the cultured or natural cyanobacteria samples collected as part of this study had detectable levels of BMAA (Table 7). Similarly, neither BMAA nor DAB were detected in animals collected from the lower Chesapeake Bay or Lake Erie regions. Both BMAA and DAB were identified and quantified in a blue crab (*Callinectes sapidus*) sample from Annapolis that had previously tested positive for these compounds (Field et al., 2013) Concentrations in replicate samples were 9.5 $\mu g\ g^{-1}$ and 5.7 $\mu g\ g^{-1}$, respectively. BMAA was not detectable in 1 or 2 mg (dry weight), but was identified in 5mg and 10mg of blue crab tissue samples. The chromatogram showed a large peak at 12.25 min., which was later identified as BMAA and a co-eluting compound, n-Propyl hexadecanoate. The compounds were separated using the extract ion chromatogram function in the ChemStation software (Fig. 7).

Table 7.
Summary of BMAA and DAB concentrations in all biological samples analyzed.

	Location	BMAA (μg g^{-1})	DAB (μg g^{-1})
Fish (Refer to Table 4 for locations)			
Quillback	-	ND	ND
Common Carp	-	ND	ND
Channel Catfish	-	ND	ND
Buffalo fish	-	ND	ND
Bluegill	-	ND	ND
White Crappie	-	ND	ND
Oysters			
Crassostrea virginica	Cape Charles, Va	ND	ND
Clams			
Mercenaria mercenaria	Cape Charles, Va	ND	ND
Blue crab			
Callinectes sapidus	Cape Charles, Va	ND	ND
Callinectes sapidus	Annapolis, Md	9.5	5.7
Cultures			
Anabaena sp.	UNC	ND	ND
Cylindrospermopsis	UNC	ND	ND
Lyngbya sp.	UNC	ND	ND
Microcystis aeruginosa	UNC	ND	ND
Synechococcus sp.	ODU	ND	ND
Trichodesmium sp.	ODU	ND	ND
Natural cyanobacteria			
Microcystis sp.	Norfolk, Va*	ND	ND
Planktothrix sp.**	Sandusky Bay	ND	ND

ND, Not Detected

* Little Creek Reservoir

**2013 and 2014 samples.

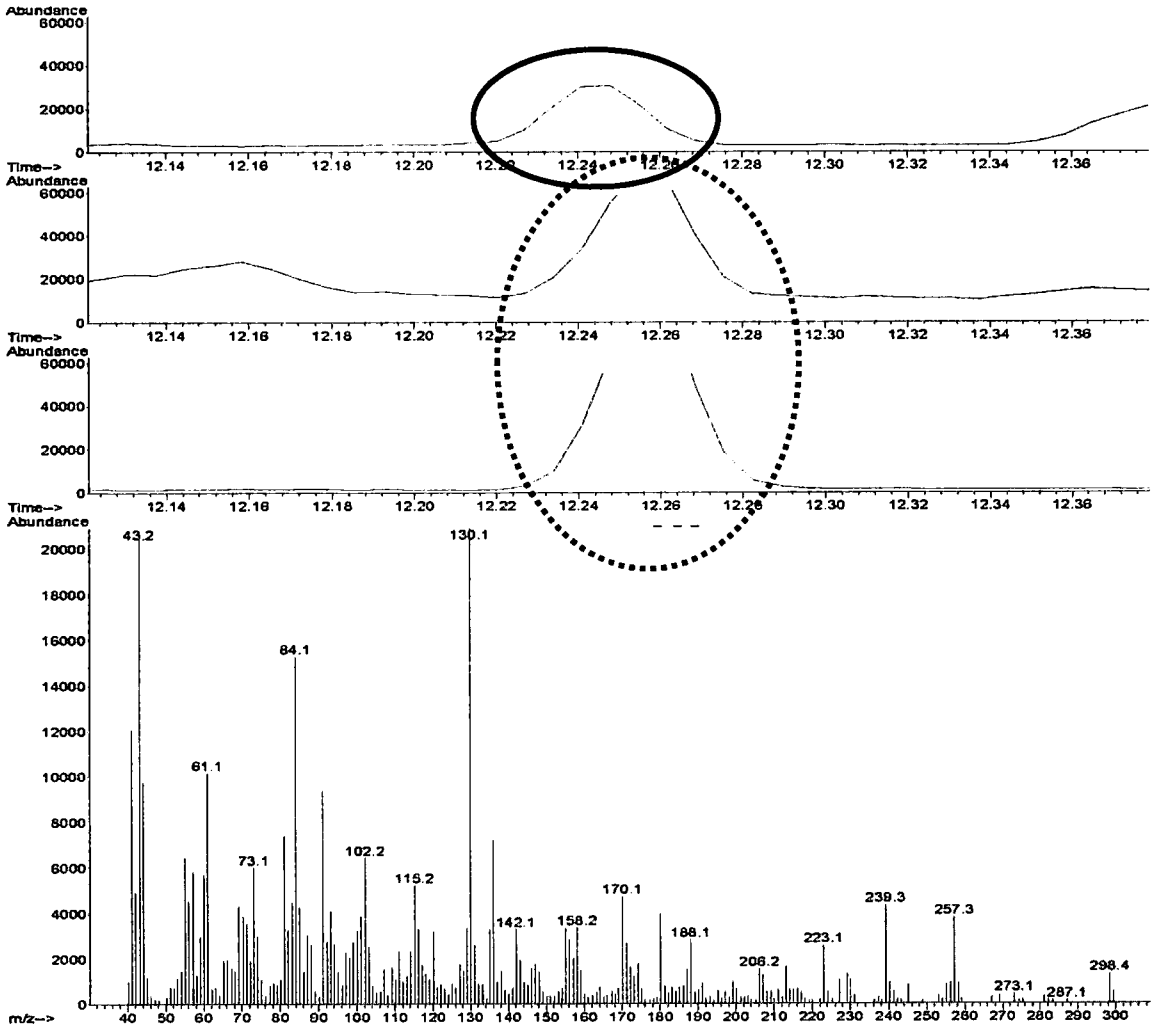

Fig. 7. BMAA peak (solid oval) and peaks of co-eluting compound (dotted oval), separated by use of EIC function. The mass spectrum is that of the BMAA peak.

2.4. Discussion

Cyanobacteria are ubiquitous in aquatic systems, and several representatives of this group produce secondary metabolites, many of which are known toxins (i.e. microcystins, saxitoxins, nodularins), that directly or indirectly affect humans (Carmichael, 1992; Falconer, 1999; Van Dolah, 2000; Dow & Swoboda, 2002; Stewart and Falconer, 2008; O'Neil et al., 2012). β-Methylamino-L-alanine (BMAA) is one of the toxins produced by many species of cyanobacteria (Cox et al., 2005; Banack et al.,

2007), and it has been linked to neurodegenerative diseases in humans. Thus, given the global proliferation of cyanobacteria blooms, and their role as primary producers in many food webs, it is vital to understand BMAA's distribution in the environment, its transmission through aquatic food webs, and potential routes of exposure to humans.

Cyanobacteria blooms have increased in recent decades as a result of cultural eutrophication (Conley et al., 2009; O'Neil et al., 2010; Paerl et al., 2011). Because this group thrives in warm, stagnant waters, increases in water temperature will likely favor the proliferation of this group in the future (Paerl et al., 2011; Newcombe et al., 2012; O'Neil et al., 2012). This could result in higher risk of human exposure to BMAA. Cyanobacteria abundance has increased in the waters of the Chesapeake Bay; specifically, the abundance of *Microcystis* spp. has risen (Marshall & Burchard, 2004). However, many other genera of cyanobacteria (i.e. *Anabaena* spp., *Lyngbya* spp., *Planktothrix* spp.), and upwards of 126 species of cyanobacteria have been identified in the Chesapeake Bay estuary (Marshall et al., 2005). Likewise, many toxic cyanobacteria genera have been identified in Lake Erie and surrounding freshwater lakes (Boyer 2008; Saxton et al., 2011), including: *Microcystis*, *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya*, *Nostoc* and *Planktothrix* (Cheung et al., 2013). While many of these genera have been identified as BMAA producers (Cox et al., 2005), their presence alone does not mean that the toxin is being produced. The present study failed to identify BMAA in samples collected during the summers of 2013 and 2014 in Chesapeake Bay and the Lake Erie regions even during a bloom of *Planktothrix* spp. in Sandusky Bay during 2014. However, sampling of plankton and aquatic animals was limited to a few sampling dates. Regular monitoring of these localities for toxins is

necessary to determine if and when BMAA is produced in these water bodies because of the extensive recreation and fishing industries that put humans in direct contact with cyanobacteria.

Although I was unable to detect BMAA in the cyanobacteria examined here, it is still plausible that these species produce this secondary metabolite. It has been thought that nutrient concentrations (i.e. nitrogen) can affect the production of cyanotoxins, including microcystins and BMAA (Bargu et al., 2011; Downing et al., 2011). Nutrient concentrations in both the Chesapeake Bay and Lake Erie region fluctuate greatly depending on seasonal nutrient inputs and mixing, runoff, and nutrient regeneration rates (Leon et al., 2005; Filippino et al., 2009; Murphy et al., 2011). Nutrient concentrations were not measured as part of this study and the physiological status of algal populations was unknown at the time of sampling. Samples analyzed as part of this study may have been collected when nutrient concentrations were not conducive for toxin production. Neither was BMAA detected in the cultured cyanobacteria analyzed during this study. The latter was unexpected, given that previous studies detected BMAA in many of the same genera of cyanobacteria (Cox et al., 2005; Esterhuizen & Downing, 2008). I did not detect BMAA in cultures of *Anabaena* spp. (Florida and New Zealand), *Cylindrospermopsis raciborskii* (Florida), *Microcystis aeruginosa* (China), or *Lyngbya* sp. Cultures analyzed as part of this study were all nutrient replete and thus conditions may not have been conducive for toxin production. In addition, there may be isolate-specific differences in toxin production even within the same species, as has been shown for microcystin production (Mikalsen et al., 2003). BMAA production has also been shown to vary greatly among different strains of the *Synechocystis* (Kruger et

al., 2010). The culture isolates used here were from different regions than those used in previous studies. In samples of natural phytoplankton communities, I was unable to identify many of the cyanobacteria down to the species level, and, thus, the specific strains analyzed may not have been those which have been previously identified as BMAA producers.

It is also unknown whether BMAA naturally degrades in the environment thereby reducing exposure risk over time. Microcystins, can be degraded through photodegredation and biological processes (Schmidt et al., 2014). It was shown that metabolic breakdown of microcystins can occur on the order of a day to a week (Schmidt et al., 2014). The timescale for breakdown due to photodegredation was longer (a few days to weeks), and the amount of time greatly depends on the light source. If BMAA does degrade, then it is probably ephemeral or seasonal in the environment. Because the samples collected during the *Planktothrix* bloom as part of this study were collected all at one time, the window of BMAA production or presence in the environment could have been missed.

Neither the *Planktothrix* samples nor the multiple fish species collected at the same time as *Planktothrix* samples from the Lake Erie region contained any detectable BMAA. It is possible that the strain of *Planktothrix* collected was a non-toxic strain or that it was not making toxin at the time of its collection. Because water chemistry is also known to play role in cyanotoxin production (Bargu et al., 2011; Downing et al., 2011), conditions at the time the bloom was sampled may not have been conducive to the production of BMAA, or it may have been degraded prior to sampling.

Similar to the Lake Erie region samples, BMAA was not detected in animals collected from the lower Chesapeake Bay during the summer of 2014. The absence of BMAA in the clam, oyster, and crab samples might be explained by the lack of cyanobacteria blooms in the lower Chesapeake Bay during 2014. While cyanobacteria are always present in the lower Chesapeake Bay, algal assemblages in the lower Chesapeake Bay and its tributaries are generally dominated by diatoms except during seasonal dinoflagellate blooms (Marshall et al., 2005; Marshall & Egerton, 2012). Therefore, cyanobacteria abundance may have been too low to detect BMAA in environmental samples. Little is known about a threshold of cyanobacteria abundance needed to detect BMAA. Low cyanobacteria abundances may present toxins levels too low to allow for detection, and particular strains, under varying environmental conditions, may produce the toxin in a higher concentration allowing for detection when biomass is low. Likewise, cyanobacteria blooms are known to contain multiple species (Merel et al., 2013), and if a bloom contains toxin producers and non-producers, the ability to detect BMAA and other toxins may be greatly diminished, simply due to the dilution of the toxin.

More recently, studies have shown that cyanobacteria may not be the only source of BMAA, but diatoms (Jiang et al., 2014) and dinoflagellates (Lage et al., 2014) produce the secondary metabolite as well. If BMAA is produced by these other taxa of phytoplankton, the global exposure risk to BMAA may be much greater than once thought. Further investigation is needed to determine whether dinoflagellates and/or diatoms are producers of BMAA in the lower Chesapeake Bay region as this is an area

of seasonally intense dinoflagellate blooms (Marshall et al., 2005, Mulholland et al., 2009; Marshall & Egerton, 2012).

The GC-MS method employed here showed clear separation of BMAA and DAB standards and results were confirmed using an LC-MS Orbitrap method. To determine whether the methods in this study could accurately detect BMAA, a positive sample previously analyzed in another laboratory was analyzed. Confirmation of BMAA in this sample demonstrated that the method employed in this study was suitable. Misidentification has been noted as a possible explanation for high concentrations, or even the presence, of BMAA in biological samples (Rosen & Hellenas, 2008; Kruger et al., 2010). For example, a liquid chromatograph-tandem mass spectrometer LC-MS/MS method employing 6-aminoquinolyl-n-hydroxysuccinimidyl carbamate (AQC) detection by fluorescence (Cox et al., 2005) has fallen under scrutiny because multiple compounds react with the fluorescent derivatizing reagent and retention time is used for quantification (Rosen and Hellenas, 2008). This can lead to misidentification because the BMAA isomer has a similar retention time so may not be fully separated from BMAA. Although the GC-MS method used in the present study produced a clear separation of BMAA and DAB, another compound was found to co-elute with BMAA. This compound, which was not DAB, could have easily led to misidentification if the spectrum of the entire peak were not carefully analyzed. Likewise, it has been suggested that compounds have been incorrectly identified as BMAA due to the common occurrence of the m/z 130.1 ion (Kruger et al., 2010). However, the amino acid standard used as part of the EZ:Faast^{TM} kit showed a clear

separation of essential amino acids (i.e. alanine, sarcosine, & tryptophan) that are known to have the m/z 130.1 ion.

Advances in the study of BMAA and its distribution in the environment would greatly benefit from resolution of these methodological differences and determining which methods are suitable for analyses of biological samples that have complex matrices (Faassen, 2014). It is difficult at present to compare results between laboratories because there is no standard reference material, and there have been no inter-laboratory comparisons analyzing the same samples using all the different methods and instrumentation employed to date. It would be ideal to perform inter-laboratory comparisons using common samples in order to better calibrate instruments and to determine which analytical methods are most appropriate for distinguishing BMAA from other compounds in tissue samples and other complex matrices. Because of a variety of methodological issues, many studies have failed to provide sufficient evidence that BMAA was indeed detected (Faassen, 2014). In this present study, BMAA was clearly separated from DAB using GC-MS, had a unique retention time, and could be accurately quantified using an internal standard.

While the results of the present study show that BMAA was not detectable in biological samples collected from the lower Chesapeake Bay and Lake Erie region, it is important to understand that BMAA may still exist in these localities. In the lower Chesapeake Bay, our findings were not surprising because few cyanobacteria blooms were reported during collection periods. However, failure to detect BMAA in Lake Erie region samples collected during a bloom of *Planktothrix* sp., and in cyanobacteria cultures was surprising because BMAA was previously detected in a *Planktothrix*

isolate from Northern Ireland and many other genera analyzed in the present study (Cox et al., 2005). Although our findings are contradictory to previous studies, the methodological practices employed here were able to distinguish BMAA from DAB and other co-eluting compounds. The lack of BMAA in samples analyzed may be related to some environmental factor(s) limiting its production. Similar to results presented here, other studies were also unable to detect BMAA in several strains of cyanobacteria from China, Japan, and Oregon (Kubo et al., 2008; Li et al., 2010; Kruger et al., 2010). Whether the discrepancies in results between studies are due to methodological differences or simply the fact that toxin production is highly variable warrants further investigation. Given the global increase in cyanobacteria blooms and the putative link with neurodegenerative diseases, it is paramount that we gain an understanding of the distribution of BMAA in the environment, factors controlling its production by cyanobacteria and other phytoplankton, and its transmission through aquatic food webs.

2.5 Conclusions and Future Studies

This thesis presents data regarding the presence of the cyanotoxin β-methylamino-L-alanine (BMAA) in aquatic organisms collected from the lower Chesapeake Bay estuary and the Lake Erie region (northwestern Ohio). A GC-MS method was used to quantify BMAA and results were verified using an LC-MS Orbitrap method. Even though we collected samples from two different ecosystems over two summers, we were only able to identify BMAA in one sample. The absence of BMAA in samples collected as part of this study leads to three plausible conclusions: 1) the cyanobacteria in the localities studied do not produce BMAA, 2) cyanobacteria

were not present at sufficient densities to detect BMAA in water samples or animals, and/or 3) BMAA production is regulated by environmental, metabolic, or chemical factors and was not being produced by cyanobacteria during this study. Further monitoring of BMAA in natural and cultured cyanobacterial populations is necessary to understand controls on the production of this toxin and the risks of human exposure.

While methodological controversy abounds regarding results reported in the literature to date, we believe our results are robust because BMAA was differentiated from co-eluting compounds, including its isomers, in all of our samples. Further, the method employed here did detect and quantify BMAA in a sample that previously tested positive for BMAA (Field et al., 2014). It is recommended that the LC-MS Orbitrap method employed here be refined further to ensure accurate detection and quantification of BMAA. I believe studies of the distribution and persistence of BMAA in aquatic organisms would benefit the most by the development and implementation of methodological standards and extensive inter-laboratory comparisons and collaboration. Although this is not possible at present, resolving methodological differences and discrepancies in results between laboratories would help to eliminate, or at least diminish, any controversy surrounding BMAA studies.

There are many intriguing avenues for the advancement of BMAA studies. For example, more work is needed to determine why and under what conditions cyanobacteria produce this compound. One recent study suggested that BMAA is produced under nitrogen-limited conditions and serves as a source of nitrogen for the cyanobacteria (Downing et al., 2011). However, there have been no other studies examining whether BMAA is always being produced by cyanobacteria, or if specific

environmental and/or chemical factors trigger its production. Further investigation is needed to determine the benefit of BMAA production to cyanobacteria, and its effects, if any, on the aquatic organisms that consume BMAA. In addition to its production, nothing is known about whether BMAA naturally degrades in the environment, and how long it persists in cyanobacterial cells or tissues of the animals that consume them. This is important for determining the safety of seafood that has been exposed to BMAA through dietary pathways. If BMAA is short-lived in cyanobacterial cells or the tissue of animals that consume them, then it may be possible to protect humans from exposure by closing fisheries sporadically, only when BMAA is being produced and consumed by grazers. Given the toxicity of BMAA, regulations on contaminated seafood might be necessary to protect human health in areas where seafood is a dietary staple. Overall, there is much uncertainty surrounding BMAA, and further research, as aforementioned, is necessary to fully understand the distribution and persistence of this compound in the environment, and its threat to human health.

CHAPTER 3

SUMMARY AND CONCLUSIONS

There are many plausible explanations as to why BMAA concentrations were below detectable limits in the present study. The first possible reason could be that the cyanobacteria may not have been producing the toxin, either due to their physiological status or environmental factors (i.e. nutrients and temperature). Secondly, it is possible that BMAA was excreted or broken down before the animals were collected. Little is known about controls on BMAA production by cyanobacteria or its residence time in cyanobacteria, water, or animal tissue. However, microcystins are a more widely studied group of cyanotoxins whose production is known to be affected by nutrient concentrations, temperature, light intensity, and the physiological status of cells (Sivonen et al., 1990; Orr & Jones, 1998; Wiedner et al., 2003; Orihel et al., 2012). Amino acids and peptides, including microcystins, are known to be degraded naturally in the environment through microbial or photodegradative pathways (Baker, 1981; Schmidt et al., 2014), or eliminated from animals that consume them via excretion or cellular metabolism or catabolism (Ozawa et al., 2003) . Little is known about how BMAA production is affected by the above mentioned factors, or how it is degraded in water or in animal tissue. Based on the findings of the present study, it is likely that either the toxin was not being produced at the time of sampling or that one or more factors known to affect toxin production or degradation reduced toxin concentrations below detectable levels.

3.1. Effects of nutrient concentrations on toxin production

There is little known about how BMAA production is affected by nutrient concentrations or other factors affecting cyanobacterial growth and metabolism. One study suggests that BMAA production increases when *Microcystis* cells are starved of nitrogen (Downing et al., 2011). However, this study only examined one strain of *Microcystis*, and a single limiting nutrient (nitrogen), and did not quantify BMAA concentrations rather reported relative peak intensity. Other nutrients (i.e. phosphorous) or growth factors (e.g., light, temperature, trace element supply), might also inhibit or promote BMAA production by cyanobacteria, but they have yet to be tested. More is known about the effects of nutrient concentrations on the production of microcystins, which are a group of hepatoxins produced by a few genera of cyanobacteria (i.e. *Microcystis, Oscillatoria,* and *Anabaena*) (Carmichael 1992). *Oscillatoria agardhii* produced more microcystins when nitrogen concentrations were high (Sivonen 1990). It was also shown that when nitrogen and phosphorus were both high, microcystin production increased in two strains of *Microcystis* (Vezie et al., 2002). Another study found that microcystin concentrations in several Canadian lakes were greatest when the nitrogen to phosphorous ratios (N:P) were low and below 23 (23:1, N:P), and sharply decreased with increases in the amount of nitrogen relative to phosphorus (Orihel et al., 2012). Based on the sensitivity of microcystin production to nutrient concentrations, it is likely that nitrogen and/or phosphorus concentrations could also affect the production of BMAA. It is possible therefore that BMAA was not being produced by cyanobacteria in the present study because nutrient concentrations were too high or low in the locations sampled during this study. Nutrient concentrations in both the Chesapeake Bay and Lake Erie region fluctuate greatly depending on seasonal nutrient

inputs, mixing, runoff, and nutrient regeneration rates (Leon et al., 2005; Filippino et al., 2009; Murphy et al., 2011). Nutrient concentrations were not measured at the time when samples were collected for the present study, and cyanobacteria cultures were growing under nutrient replete conditions. Monitoring nutrient and toxin concentrations simultaneously in environmental samples is necessary to better understand the relationship between nutrient concentrations and BMAA production in natural waters.

3.2. Effects of water temperature on toxin production

Water temperature also affects cyanobacterial growth and may affect the production of BMAA. However, unlike nutrients, temperature has not been studied as a factor controlling BMAA production. Temperature has been shown to affect the growth of toxic and nontoxic *Microcystis* strains. Growth of toxic strains of *Microcystis* increased by 83% when grown at higher temperatures (+4°C); however the growth of nontoxic strains only increased 33% (Davis et al., 2009). Although toxin levels were not measured as part of this study, if toxin production increased proportionally with temperature then warmer temperatures may result in higher toxicity. The results of this study were used to infer that increases in temperature due to global warming could increase the production of toxic strains of *Microcystis* and the amount of toxin that organisms are exposed to (Davis et al., 2009). Contradictory to those findings, the production of microcystins by *Oscillatoria agardhii* were highest when growing at average temperatures of 15-25°C and decreased when temperatures were at or over 30°C (Sivonen et al., 1990).

In the present study, water temperatures ranged from 21.05 to 29.33 °C during the period of sample collection in the in the lower Chesapeake Bay (Chesapeake Bay

Interpretative Buoy System Data (NOAA)). Water temperatures in the Sandusky Bay in September 2013 averaged 21.4 °C and temperatures in July 2014 averaged 22.2 °C (National Weather Service). It is impossible to speculate on whether water temperature affected BMAA production at the sites sampled because for the present study sampling was limited to a few points in time. Samples should be collected over seasonal cycles as temperatures warm and then cool to determine whether water temperature is an important control on BMAA production in the Lake Erie and the lower Chesapeake Bay regions. Nutrient concentrations and temperature affect the production of microcystins, and given the seasonal fluctuations of those two parameters, further studies on the individual and interactive effects of temperature and nutrient concentrations on the production of BMAA are necessary.

3.3. Effects of light intensity on toxin production

Light intensity may also play a role in cyanotoxin production. Cellular microcystin content was positively correlated with growth rate under light-limited conditions (photosynthetically active radiation (PAR) of 10 and 40 μmol photons $m^{-2}s^{-1}$). Maximum microcystin concentrations of 81.4 fg $cell^{-1}$ were recorded at 40 μmol of photons $m^{-2}s^{-1}$ compared to 34.5 fg $cell^{-1}$ under light-replete conditions (Wiedner et al., 2003). Although light intensity may affect toxin production, it was not measured during collection of samples as part of the present study. However, for the present study, cultured cyanobacteria were maintained under a 12:12 h light:dark cycle under a constant light supply (11.08 μmol photons $m^{-2}s^{-1}$). It has been shown that cellular microcystin concentrations are higher at a similar intensity (10 μmol photons $m^{-2}s^{-1}$) (Wiedner et al., 2003), and if BMAA production is affected by light intensity in the

same manner, the culture conditions for the present study were ideal. However, extracellular (dissolved) microcystin levels were 20 times higher at PAR of 40 μmol photons $m^{-2}s^{-1}$ (Wiedner et al., 2003). The extracellular BMAA concentrations were not analyzed for the present study, nor were the PAR levels increased to 40 μmol photons $m^{-2}s^{-1}$. The findings of Wiedner *et al.* (2003) suggest that light intensity should be considered as a potential factor affecting BMAA production.

3.4. Other physiological factors affecting toxin production

Although the effects of the physiological status of cyanobacteria cells on BMAA production have not been examined, it was shown that there was a positive linear relationship between microcystin production and growth rate for *Microcystis* (Orr & Jones, 1998). This suggests that the physiological state of cyanobacteria may affect toxin production. Given that algal growth in cultures is described by distinct phases (i.e. lag, exponential, stationary growth phases), and the physiological status of algal cells in natural systems changes as a result of environmental variability and as blooms initiate, develop, and senesce, the findings by Orr and Jones (1998) suggest that toxin production may be highest when cells are growing exponentially. The growth phase and physiological status of natural and cultured cyanobacteria sampled during the present study was not determined. However, the Lake Erie samples were collected when algal biomass was high, the bloom was well-established, and just before bloom demise and thus the cyanobacterial population may have been in post-exponential phase growth. Future sampling of natural cyanobacterial populations should be done during all growth/bloom stages to relate physiological state of populations to BMAA production.

3.5. Species variability and toxin production

The production of BMAA may be highly species and/or strain dependent. For example, BMAA production was highly variable among species of the same genera and strains of the same species (Table 8) (Cox et al., 2005; Kruger et al., 2010). These findings suggest that the production of BMAA is limited to certain species or strains of cyanobacteria. However, it is also possible that the environmental factors or physiological state of the populations sampled, as mentioned above, caused the disparities between the two studies.

Table 8.
Summary of BMAA concentrations reported by Cox *et al.* (2005) and Kruger *et al.* (2010)

	BMAA Concentrations µg g^{-1}	
Species	Kruger *et al.* (2010)	Cox *et al.* (2005)
Cylindrospermopsis raciborskii	ND	6492
Microcystis aeruginosa	ND	10
Nodularia harveyana	ND	31
Synechocystis spp.	ND	ND
*Anabaena*spp.	ND	35

3.6. Removal or catabolism of toxins, including BMAA

During and following cyanotoxin production, animals and humans may be exposed to these compounds through drinking water containing dissolved toxins, aerosolized toxins, or through ingestion of organisms which have consumed the cyanobacteria that produce the toxins. The rate at which toxic amino acids degrade is very important to understand because the shellfish and fishing industries rely heavily on harvesting and selling non-contaminated products. Cyanotoxins can be released

extracellularly or through cell lysis, and pose a risk to humans or animals that drink the contaminated water (Merel et al., 2013). Once the toxin is in the water, the compounds are often broken down through photo or microbial degradation (Baker et al., 1981; Park et al., 2001; Schmidt et al., 2014). Microcystins can be photodegraded, and the time it takes (days to weeks) to degrade in the environment depends on the type and amount of light to which they are exposed (Schmidt et al., 2014). This breakdown of amino acids is often due to microbial breakdown after release of the toxin into water following cell lysis (Baker 1981). There are no studies that have examined the degradation of extracellular BMAA; however, the natural degradation of microcystins in natural waters and animal tissue has been widely studied. Bacterial species, such as *Pseudomonas aeruginosa and Sphingomonas* sp., degrade microcystins following their release during cell lysis (Jones & Orr, 1994; Park et al., 2001). These bacteria are known to break down microcystins on time scales of days to weeks in natural environments suggesting that exposure risk, mainly to organisms affected by drinking the water, through contact with dissolved microcystins is limited to that length of time (Jones et al., 1994; Park et al., 2001). It is possible that the cyanobacteria collected for the present study were producing BMAA, but due to bacterial or photo degradation, the toxin concentrations were degraded below detectable concentrations.

Most aquatic animals are thought to be exposed to cyanotoxins via ingestion of the cyanobacteria producing them. Cyanotoxins may be naturally excreted from the tissues of aquatic organism that consume the toxins. Results show that microcystins decreased naturally in the snail (*Sinotaia histirca*) at a rate of 0.0828 day^{-1}, and had a biological half-life of 8.4 days (Ozawa et al., 2003). It is possible that BMAA was

excreted from the tissues of the organisms collected for the present study to concentrations below detection limits; however, no data is available concerning the potential emission of BMAA from the tissues of organisms.

Female blue crabs (*Callinectes sapidus*) migrate from the northern, lower salinity waters of the Chesapeake Bay to the higher salinity southern portions of the estuary in order to spawn (Turner et al., 2003; Aguilar et al., 2005). It is possible that the crabs were exposed to BMAA producing cyanobacteria in the northern portions of the Bay, as shown by the BMAA positive sample from Annapolis, Maryland (Field et al., 2013). Following mating, female blue crabs tend to forage at high rates in order to acquire energy for migration (Turner et al., 2003). It is possible that the blue crabs collected by Field *et al.* (2013) were obtained during periods of high foraging, during a bloom, and large amounts of the toxin were ingested prior to analysis (Field et al., 2013). It is possible that the blue crabs collected for the present study were not consuming the cyanobacteria at high enough rates to incorporate detectable concentrations of BMAA in their tissues. Also, the crabs collected during the present study may have been exposed to BMAA during their migration, but the timing of migration may have been long enough for BMAA depuration or excretion prior to analysis.

The sex of the crabs was not reported by Field *et al.* (2013), but it was not a factor in the results of the present study, given that both females and males had BMAA concentrations below detection limits. However, the sex was not a determining factor for which samples to collect and the amount of each sex varied with collection dates. Females generally migrate more than males (Turner et al., 2003; Aguilar et al., 2005),

and they are potentially exposed to areas with varying algal communities and environmental conditions. If BMAA production is strain dependent or affected by environmental conditions, females may have a higher chance of being exposed to cyanobacteria which produce BMAA. Future studies should monitor the sex of blue crabs in order to better understand if BMAA content is linked to a particular sex.

The clams (*Mercenaria mercenaria*) and oysters (*Crassostrea virginica*) analyzed from Cherrystone Aqua-farms are sedentary organisms, meaning that in order for them to incorporate BMAA into their tissue, the toxin producing cyanobacteria must bloom within a relatively short distance. If cyanobacteria abundance was not great enough, the clams and oysters will not ingest the compound simply due to lack of exposure. There is no published data on the cyanobacteria abundance in the lower Chesapeake Bay during the collection periods of this study (Summer 2014). Likewise, cyanobacteria blooms are not prominent in the estuarine waters of the Bay, which are dominated by blooms of diatoms and dinoflagellates (Paerl & Otten, 2013). The fact that cyanobacteria blooms are not prevalent in the Chesapeake Bay, may eliminate the possibility of detecting BMAA in the cyanobacteria or other organism in its waters. However, the absence of BMAA in the cyanobacteria bloom collected from Little Creek Reservoir suggests that another factor may be limiting its production.

3.7. Conclusions

BMAA has been found associated with many cyanobacteria genera as well as incorporated into proteins of cyanobacteria consumers (Cox et al., 2005). However, the concentrations within replicates and amongst studies are highly variable, ranging from 0 to nearly 7000 $\mu g\ g^{-1}$. This variability may be due in part to one or more of the

environmental or physiological factors, as mentioned above, acting on the production of BMAA. The inconsistencies may also be due to the natural degradation or excretion of BMAA prior to or following sample collection, leading to toxin concentrations which may not be truly representative of the overall production of BMAA. The lack of BMAA in the samples collected for the present study may also be due to one or more of the aforementioned factors. However, since little is known of how BMAA responds to these factors, it is impossible to speculate that one specific factor affected the results of the present study. Future studies should explore the factors limiting BMAA production and the degradation of BMAA in order to better understand human exposure potential to this putative toxin.

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