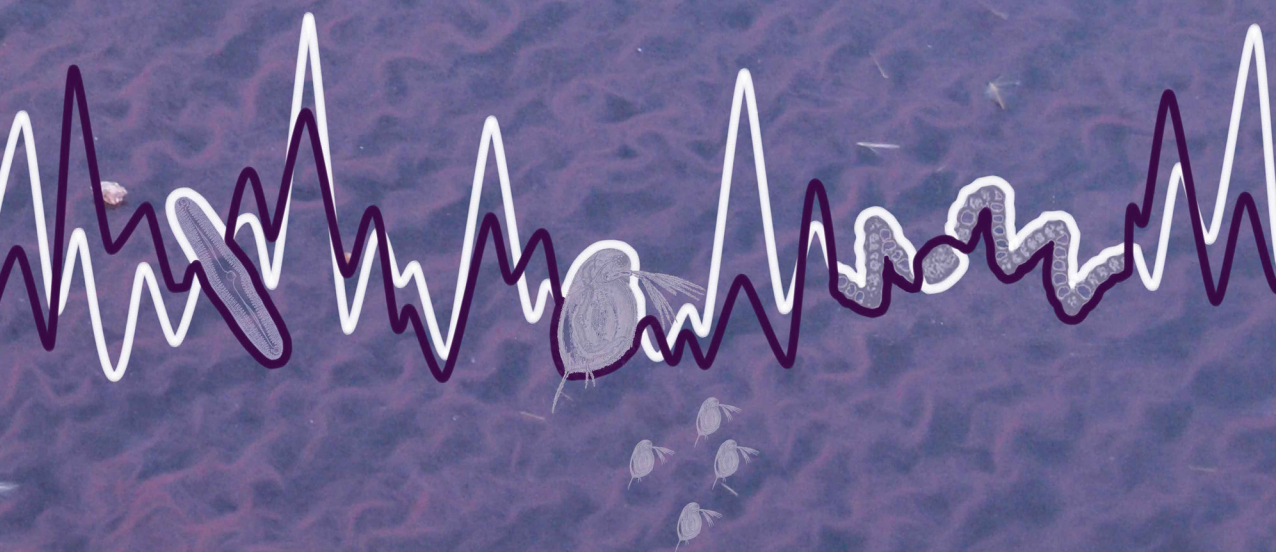# The neurotoxin BMAA in aquatic systems

analysis, occurrence and effects

Elisabeth J. Faassen

The neurotoxin BMAA in aquatic systems
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"Forget injuries, never forget kindnesses"

Confucius

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"Nobody said it was easy
No one ever said it would be so hard
I'm going back to the start"

Coldplay, The Scientist
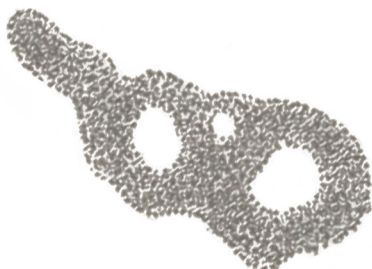
CHAPTER 1

GENERAL INTRODUCTION

1.1 EUTROPHICATION AND PHYTOPLANKTON BLOOMS

Aquatic systems are the habitat of many species and provide important ecosystem services, such as provision of drinking water and food, and coast protection [1, 2]. However, in many regions in the world, aquatic systems suffer heavily from increasing human pressure [3] and as a conse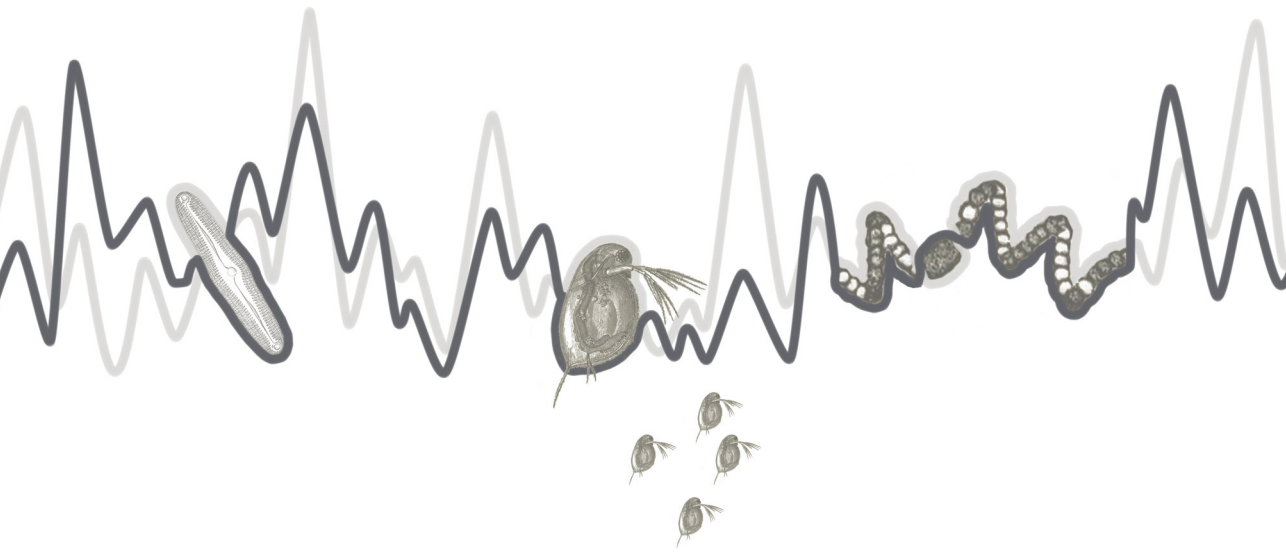quence, ecosystem functioning and biodiversity have decreased [4, 5]. Eutrophication, the pollution with nutrients, is one of the drivers of aquatic system degradation and is regarded as one of the most important water quality issues in both freshwater and marine systems [6, 7].

Eutrophication changes food webs, as primary producers, and mostly phytoplankton species, benefit from the high level of available nutrients. Zooplankton can control excessive phytoplankton growth by grazing and by doing so, they transfer nutrients and energy to higher trophic levels [8]. However, when zooplankton grazing is reduced, for instance because zooplankton is intensely preyed upon by fish [8], or because of the phytoplankton's toxicity, hard to handle morphology or poor nutritional value [9], phytoplankton can proliferate. In this situation, the trophic coupling between phyto- and zooplankton is distorted [10], and phytoplankton may reach very high densities. In freshwater systems, such phytoplankton blooms mainly consist of cyanobacteria, which can accumulate at water surfaces and lee-side shores in thick scums [11], while in marine systems, diatoms and dinoflagellates are the main blooming species (Figure 1.1). Excessive phytoplankton growth can reduce water transparency, reduce macrophyte growth and may cause anoxia and fish kills at night or upon decay [6, 7, 12]. Moreover, as will be explained in the next paragraph, phytoplankton blooms can be dangerous to humans, pets and wildlife because some phytoplankton species can produce potent toxins. Blooms of toxic species and blooms that have other detrimental effects on aquatic systems or ecosystem services are therefore often referred to as harmful (algal or cyanobacterial) blooms [6, 13, 14].

1.2 PHYCOTOXINS

One of our main concerns with phytoplankton blooms, as outlined above, is that they can be toxic. A variety of marine and freshwater phytoplankton species are capable of producing so called phycotoxins. In marine systems, phycotoxins are mainly produced by dinoflagellates and diatoms whereas in freshwater ecosystems, most toxins are produced by cyanobacteria [13]. Phycotoxins differ greatly in their structure, mode of action and level of toxicity. For instance, some toxins are protein phosphatase inhibitors (microcystins [15], okadaic acid [16]), while others block sodium channels (saxitoxins [17]), inhibit acetyl-choline esterase (anatoxin-a(s) [18]) or bind to glutamate receptors (domoic acid [19]). Main targets of phycotoxins are the nervous system and organs like liver, kidney and skin [11, 19, 20] and some toxins are tumour promotors [20, 21]. The effects of algal toxin exposure can range
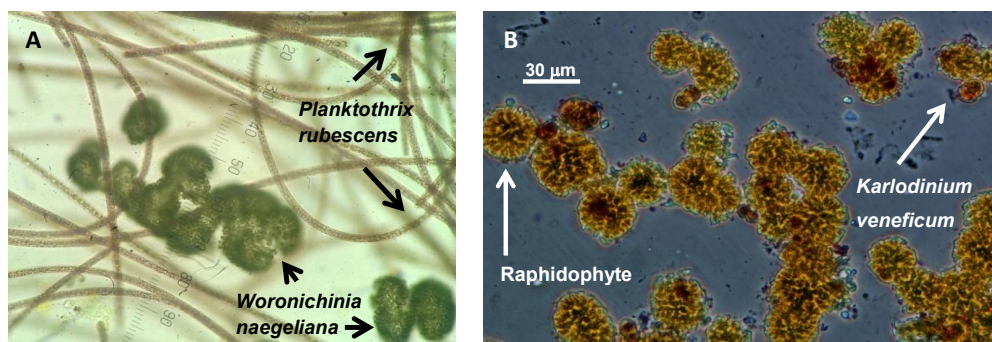
Figure 1.1. Two freshwater cyanobacteria (A, *Planktothrix rubescens* and *Woronichinia naegeliana*) and two marine blooming phytoplankton species, a raphidophyte and the dinoflagellate *Karlodinium veneficum* (B, preserved in Lugol's solution). Credit: Miquel Lürling (A) and Nathan S. Hall (B).

from mild - like nausea and local numbness - to severe, such as respiratory difficulties, paralysis and death [19, 21-23].

Many phycotoxins can be transferred from phytoplankton to higher trophic levels such as shellfish, zooplankton and fish. Humans are therefore not only exposed to phycotoxins through direct contact with toxic algal blooms, but ingestion of contaminated food can be a major exposure route as well [13, 24]. Yearly, phycotoxins are responsible for over 60.000 human intoxications worldwide [25]. Moreover, phycotoxins can kill wildlife (e.g. [26, 27]) and pets (e.g. [28, 29]). Phytoplankton blooms also have a negative economic impact: in Europe, marine blooms alone are estimated to yearly cause a 813 M€ economic loss, mainly to recreation and tourism (637 M€) and commercial fisheries (147 M€) [30].

Although hundreds of algal toxins have been identified already, new compounds are still being discovered. One of the compounds that have recently been added to the list of known phycotoxins is the non-proteinogenic, neurotoxic amino acid β-*N*-methylamino-L-alanine (BMAA, Figure 1.2). BMAA was discovered in 1967 in a terrestrial ecosystem, in the seeds of the cycad *Cycas micronesica* on the island of Guam [31]. In 2003, BMAA was found in the cyanobacterium *Nostoc* living in symbiosis with this cycad [32]. This first report of BMAA in a cyanobacterium was followed by positive reports for BMAA in free living and symbiotic cyanobacteria, as well as in cyanobacteria dominated field samples [33-36]. Given the putative role of BMAA in neurodegenerative illnesses [37], these findings of widespread occurrence of BMAA in virtually all tested cyanobacteria at sometimes alarming high concentrations led to the assumption that BMAA may pose a worldwide significant risk to human health [34, 38, 39].
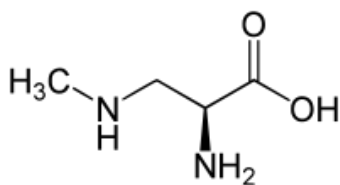
Figure 1.2. Chemical structure of BMAA.

1.3 THE ROLE OF BMAA IN THE AETIOLOGY OF NEURODEGENERATIVE DISEASES

BMAA research started on Guam [31], in search for a cause of the high incidence of amyotrophic lateral sclerosis-Parkinsonism dementia complex (ALS-PDC), a combination of amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and Alzheimer's disease (AD), among the indigenous Chamorro people. ALS-PDC incidence among the Chamorro people was about 100 times higher than in the continental United States [40], and was related to the Chamorro traditional diet, of which cycad seeds were a main constituent [41]. BMAA was found in dietary items such as cycad flour that was prepared from washed seeds, and flying foxes, a bat species that foraged on cycad seeds and that was regarded as a delicacy by the Chamorro people [42, 43]. In subsequent studies, BMAA was shown to be neurotoxic (e.g. [31, 44]), and BMAA exposure is at present regarded as one of the possible causes of ALS/PDC on Guam [45].

BMAA research expanded beyond Guam when its presence was reported in free living cyanobacteria originating from all over the world [33]. This finding implied that human exposure to BMAA could occur globally, and not only in the few ALS/PDC hotspots in the western Pacific, where the use of cycads was integrated in the traditional way of living [46-48]. BMAA exposure was now suggested to (also) play a role in the globally occurring neurodegenerative diseases AD, PD and ALS [37].

AD, PD and ALS are fatal, age-related, progressive neurodegenerative diseases. AD and PD have a high incidence: in the United States, 4.5 million people were suffering from AD in 2005, and AD incidence is expected to increase in the US to 11-16 million cases in 2050 [49]. Approximately one million Americans were affected by PD in 2005, and this number is expected to increase to 4 million in 2040 [49]. AD is the most common form of dementia [50] and this disease is characterised by the loss of neurons and synapses in the brain, mainly in the cerebral cortex [51]. PD is the second most prevalent neurodegenerative disease (after AD), and patients show tremor at rest, rigidity, slowness or absence of voluntary movement, instability and freezing [52]. PD is characterised by loss of neurons in the substantia nigra, a part of the mid brain [52]. ALS, of which patients have a life expectancy of 3 to 5 years after diagnosis [53], is characterised by motor neuron degeneration, leading to spasticity,

hyperreflexia, weakness, muscle atrophy and paralysis. ALS does not affect the heart muscle, and most patients die from respiratory muscle failure [54]. These three neurodegenerative diseases have in common that for most of the cases (> 99% in AD [50] and approximately 95% in PD [52] and ALS [53]) the disease is not inherited and at present, it is still unknown what triggers the onset of disease in these sporadic cases. Another common feature is that in all three of these diseases, excitotoxicity (neural injury or death invoked by excessive activation of glutamate receptors [55]), oxidative stress and protein misfolding are involved [56, 57].

BMAA exposure is one of the possible environmental factors that could play a role in the aetiology of AD, PD and ALS. Possible human exposure routes to BMAA include exposure to BMAA-containing phytoplankton, or through ingestion of higher aquatic organisms that have been in contact with such blooms [58]. The neurotoxicity of BMAA has been recognized both *in vitro* and *in vivo* [59, 60], and BMAA-mediated excitotoxicity and oxidative stress have been experimentally demonstrated (e.g. [61-64]). Furthermore, there is some indication that BMAA incorporation leads to protein misfolding *in vitro* [65], but this area needs further research. But even though it is established that BMAA is a neurotoxin, an animal model for BMAA-induced neurodegenerative diseases is still lacking [60, 66]. Recently, some progress has been made with a rat [67] and an primate model [68], but further work in this field is still required.

The hypothesis that BMAA may trigger ALS, AD and PD is supported by the reports of BMAA in brains of deceased ALS/PDC and AD patients [69-71], but these results could not be reproduced by other research groups [72-74]. In a recent study on BMAA in cerebrospinal fluid, BMAA was detected in one ALS patient, but higher concentrations were found in two control patients [75]. Although the results of this study are not conclusive yet, this type of work is promising as biomarkers for BMAA exposure could be developed from *ante mortem* analyses. These biomarkers could be used in larger scale epidemiological studies with living patients, which could shed more light on the role of BMAA exposure in the aetiology of ALS, AD and PD.

1.4 CONTROVERSY REGARDING THE PRESENCE OF BMAA IN AQUATIC ECOSYSTEMS

The initial report of high BMAA levels (up to a few mg/g DW) in nearly all tested free living cyanobacteria from over the world [33] promoted considerable follow-up research. The first follow-up studies on cyanobacterial isolates and field samples confirmed the presence of BMAA in the majority of the tested samples, albeit at slightly lower concentrations than initially reported [34-36]. However, other laboratories could not detect BMAA in any cyanobacterial sample (e.g. [76-79]), although the analytical methods used were in most cases sensitive enough to enable reproduction of these first results. The claim that

BMAA was produced by symbiotic cyanobacteria in the cycad's coralloid roots [32] was challenged when cycads devoid of symbiotic cyanobacteria were found to increase in BMAA concentrations [80, 81]. This finding, in combination with the negative reports for BMAA in cyanobacteria by some research groups, raised doubts on the hypothesis that BMAA was a common cyanobacterial product.

When BMAA research expanded from cyanobacteria to higher trophic levels, similar differences between studies were observed. As an example, BMAA concentrations reported for food webs in South Florida (high µg/g up to mg/g dry weight levels [82]) were orders of magnitude higher than those reported for the Baltic Sea (mostly ng/g dry weight [83]). The cause of these conflicting results was attributed to the different analytical methods used, although there was initially little consensus on what would be the most suitable method for BMAA analysis (e.g. [77, 84]). The crucial role that analytical chemistry has played in BMAA research was clearly described in a 2012 tutorial: *"The BMAA hypothesis relies on analytical data at every twist and turn, but there are many deficiencies in the work that has been performed. The putative link between cycads and neurodegenerative disease remains highly controversial, in large part because of discrepancies in the analytical findings relating to pivotal elements of this theory"* [85]. In order to generate reliable data on the presence of BMAA in aquatic systems and in neurodegenerative disease patients, sound analytical methods should be applied, and the development of these methods starts with a thorough understanding of the analyte of interest.

1.5 BMAA ANALYSIS

1.5.1 Chemical properties of BMAA

BMAA is a small, polar compound with a molecular weight of 118.1 Da. BMAA is a basic amino acid, with pK values of 2.1 (carboxyl group), 6.5 and 9.7 (both amino groups) and a pI of 8.1 [86]. At physiological pH (7.4), the main fraction of BMAA will be a zwitterion [86]. At physiological pH and in the presence of bicarbonate, BMAA forms a carbamate that structurally resembles the neurotransmitter glutamate, which might explain why BMAA can activate glutamate receptors under physiological conditions [61] (Figure 1.3). BMAA can form complexes with metals, metal complexation is most pronounced at pH > 5 [87, 88]. BMAA does not have a chromophore and is colourless in solution.

In addition to the free molecule described above ("free BMAA") BMAA can also be present in bound forms in natural samples (Figure 1.4). The precursors of these bound forms still need to be identified. The structure of soluble bound BMAA is unknown, but in mussels, it appears to be a low molecular weight (< 3000 Da) compound in which BMAA is not bound with covalent peptide bonds [89]. And although precipitated bound BMAA is often referred to as "protein-associated" BMAA, the exact nature of the association of BMAA with proteins in natural tissues also needs to be elucidated. *In vitro*, BMAA can be incorporated into
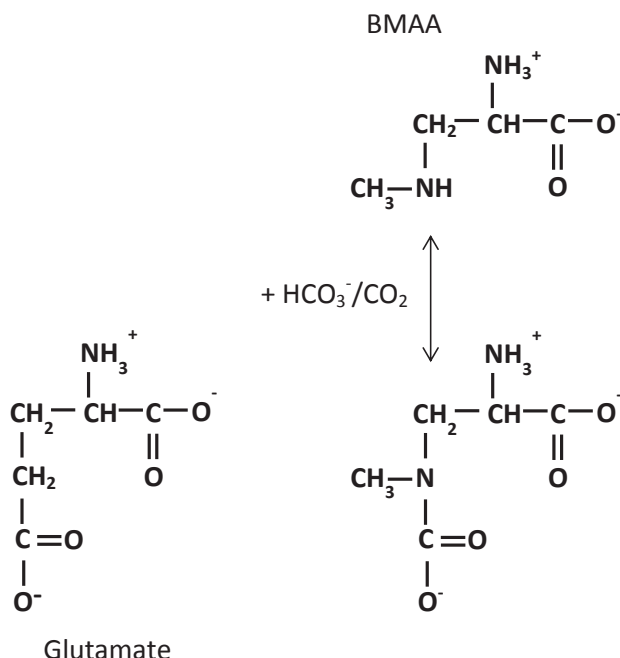
BMAA

$$NH_3^+$$
$$CH_2-CH-C-O^-$$
$$CH_3-NH \qquad O$$

$$+ HCO_3^-/CO_2$$

$$NH_3^+ \qquad\qquad NH_3^+$$
$$CH_2-CH-C-O^- \qquad CH_2-CH-C-O^-$$
$$CH_2 \quad O \qquad CH_3-N \quad O$$
$$C=O \qquad\qquad C=O$$
$$O^- \qquad\qquad O^-$$

Glutamate

Figure 1.3. A carbamate adduct of BMAA resembles glutamate. Based on [61].

proteins [65, 90], but *in vivo* experiments with bacteria do not show protein incorporation [91]. Whether protein incorporation also occurs *in vivo* is therefore still to be determined.

Free BMAA is commonly extracted with aqueous solvents, typically 0.1 M trichloroacetic acid (TCA) [70]. When the dried extract is hydrolysed, total soluble BMAA is obtained [92]. Finally, precipitated bound BMAA can be extracted by acid hydrolysis of the precipitate [70]. Total BMAA content, irrespective of its original form, is usually obtained by hydrolysis of the total sample (e.g. [93, 94]).

1.5.2. Analytical methods for BMAA analysis

Detection methods for BMAA in human tissue and environmental samples have evolved over the years and have been heavily debated (e.g. [77, 84, 88, 95]). In the past ten years, most analytical methods employed relied either on optical detection or on mass spectrometry (MS) detection [58]. As BMAA does not contain a chromophore, most methods based on optical detection, such as liquid chromatography coupled to fluorescence detection (LC-FLD), require pre-column derivatisation with a chromophore containing reagent such as 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC, e.g. [34, 96]). This pre-column derivatisation has an additional advantage: the derivatised molecule is bigger and more hydrophobic, which facilitates reversed phase LC separation. A disadvantage of optical
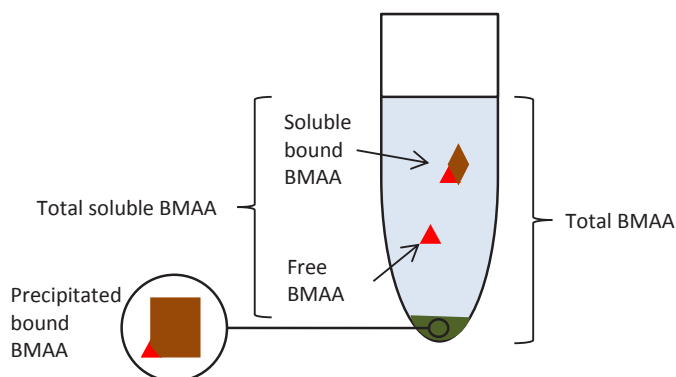
Figure 1.4. Different BMAA fractions in an aqueous extract. Free and soluble bound BMAA are found in the extract. Hydrolysis of the dried extract yields total soluble BMAA. Precipitated bound BMAA is found in the pellet created during extraction and can be released by hydrolysis. Total BMAA is the sum of all fractions and is usually obtained by hydrolysis of the total sample.

detection methods is that they are non-selective for BMAA, and therefore risk BMAA misidentification and overestimation [93]. In Box 1.1, the principles of the LC-FLD method using AQC derivatisation are explained.

Another approach to BMAA detection is mass spectrometry, and within this technique, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is mostly applied for BMAA analysis [58]. When properly applied, LC-MS/MS is more selective than optical detection techniques [58, 93]. Although pre-column derivatisation of BMAA is not required for LC-MS/MS analysis, it is often used to enhance reversed phase separation. Commonly used derivatisation techniques in combination with LC-MS/MS analysis are AQC (e.g. [34, 97, 98]), propyl chloroformate (e.g. [91, 99]) and dansyl chloride [100, 101]), see Box 1.2 for explanation of AQC derivatised LC-MS/MS analysis. LC separation of underivatised BMAA under reversed phase conditions is difficult due to the molecule's low weight and high polarity. In underivatised LC-MS/MS analysis, hydrophilic liquid interaction chromatography (HILIC) is therefore usually employed (e.g. [76, 102, 103]). Box 1.3 explains the principles of the underivatised LC-MS/MS method.

When using LC-MS/MS based methods, it is important to distinguish between BMAA and its structural isomers such as 2,4-diaminobutyric acid (2,4-DAB, mostly referred to as DAB), *N*-(2-aminoethyl) glycine (AEG), ß-amino-*N*-methylalanine (BAMA) and 3,4-diaminobutyric acid (3,4-DAB) [97, 104]. Mostly, BMAA is chromatographically resolved from its structural isomers, but additional selectivity can be obtained by more advanced techniques such as differential mobility spectrometry [104]. Isotope dilution, e.g. by addition of deuterated BMAA [76] or DAB [105], is an often applied technique to improve BMAA quantification by LC-MS/MS.

Methods such as LC-FLD and LC-MS/MS require expensive equipment and an extensive sample workup. Faster and cheaper screening of surface waters for BMAA can be done with a commercial enzyme linked immunosorbent assay (ELISA), released in 2012. The principles of this assay are described in Box 1.4. ELISA uses the specific binding between the analyte (in this case BMAA) and an enzyme-labelled antibody for quantification. If the antibody used in the assay does not only bind to the intended analyte, but also to other sample components, the assay will create false positives or will overestimate analyte concentrations. As this so called cross reactivity is a common concern in ELISA, this assay is usually used for screening purposes only, and more selective analytical methods are used to verify positive results.

1

Box 1.1. Principles of LC-FLD detection of BMAA.

Extract

Derivatised extract

Reversed phase separation

Fluorescence detection
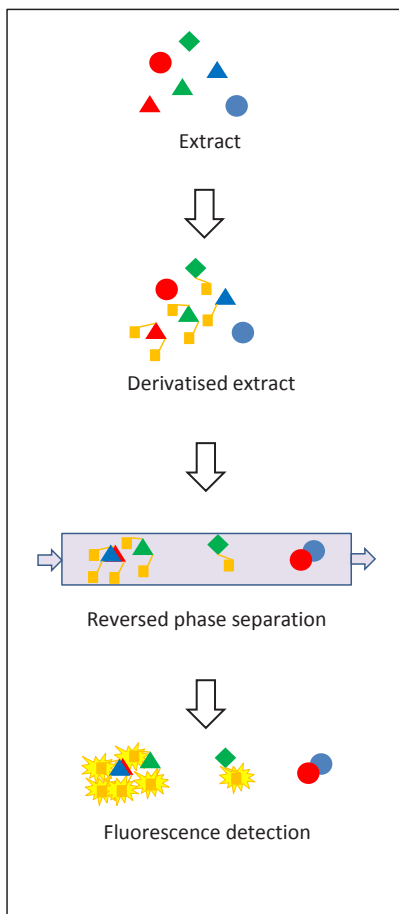
Liquid chromatography coupled to a fluorescence detector (LC-FLD) uses the fluorescent properties of analytes for detection.

First, BMAA (red triangle) is extracted to release it from the sample cells. As BMAA does not contain a chromophore, a fluorescent tag needs to be added after extraction, a process called derivatisation. In the work presented in this thesis, the reagent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) is used. AQC reacts with primary and secondary amino groups [106], and as BMAA contains two amino groups, it is double derivatised.

Derivatised BMAA is bigger and less polar than the original molecule and can therefore be separated from other matrix compounds by reversed phase liquid chromatography.

The fluorescence detector records the emission signal of the AQC tag at 395 nm after excitation at 250 nm. The emission wavelength is the same for all AQC derivatised compounds, and the method therefore only has two criteria for analyte identification: the presence of amino groups (i.e. the ability to react with AQC) and retention time. This means that selectivity of this method is low and that the chance that BMAA is misidentified or its concentration is overestimated is substantial, especially in samples in which compounds with amino groups are abundant. The green and blue triangles in the figure would in this case give an overlapping signal with BMAA and should either be removed during extraction or separated from BMAA during LC.

Box 1.2. Principles of LC-MS/MS detection of BMAA following AQC derivatisation.
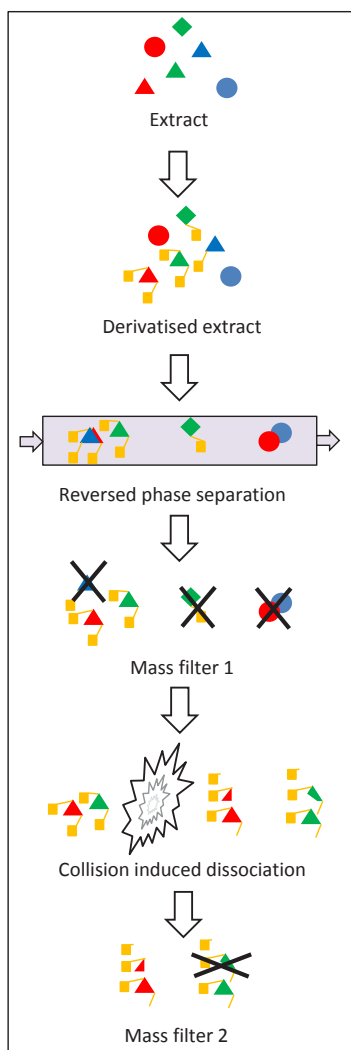
Extract

Derivatised extract

Reversed phase separation

Mass filter 1

Collision induced dissociation

Mass filter 2

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is a selective analytical method based on the on the detection of fragmented ions.

First, BMAA (red triangle) is extracted to release it from the sample cells. BMAA is then derivatised to enable reversed phase separation. In the work presented in this thesis, the reagent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) is used. AQC reacts with primary and secondary amino groups [106], and as BMAA contains two amino groups, it is double derivatised.

Derivatised BMAA is bigger and less polar than the original molecule and can therefore be separated from other matrix compounds by reversed phase liquid chromatography.

Upon entering the MS/MS, the molecules are protonated and become ions (precursor ions). In the first mass filter, only ions with the same mass-to-charge ratio (*m/z*, the mass of the molecule divided by the charge obtained after protonation) as BMAA (*m/z* 459) are selected.
The ions that pass the first mass filter are subjected to collision-induced dissociation. In the collision cell, the ions break into distinct fragments. Under the right settings, the fragmentation pattern is reproducible and unique for each compound.

In the second mass filter, only product ions (fragments) characteristic of the analytes of interest are selected based on their *m/z*, these are the fragments that are detected. In the work presented in this thesis, the product ions *m/z* 171, 119 and 258 or 145 are used for BMAA identification.

LC-MS/MS has four criteria for analyte detection: retention time, *m/z* of the precursor ion, *m/z* of the product ions and the ratio between these product ions. Compounds that have a precursor with the same *m/z* after derivatisation and that (partly) give similar product ions as BMAA (green triangle, e.g. DAB) can interfere with the BMAA signal. These compounds need to be removed during extraction or separated from BMAA by LC before entering the MS/MS.

Box 1.3: Principles of LC-MS/MS detection of BMAA without derivatisation.

Extract

HILIC separation

Mass filter 1

Collision induced dissociation

Mass filter 2

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is a selective analytical method based on the detection of fragmented ions.

First, BMAA (red triangle) is extracted to release it from the sample cells. BMAA can be separated by LC without derivatisation. As BMAA is a small, polar compound, separation by reversed phase chromatography is difficult. In the work presented in this thesis, separation of underivatised BMAA from other compounds is therefore performed by hydrophilic interaction liquid chromatography (HILIC).
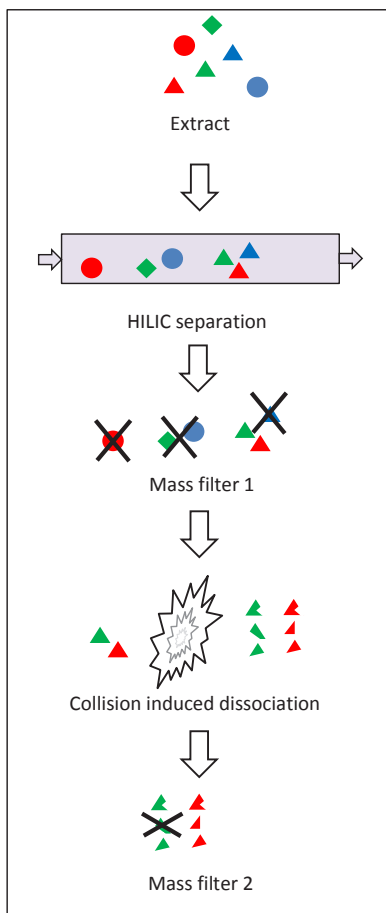
Upon entering the MS/MS, the molecules are protonated and become ions (precursor ions). In the first mass filter, only ions with the mass-to-charge ratio (m/z, the mass of the molecule divided by the charge obtained after protonation) of BMAA (m/z 119.1) are selected.

The ions that pass the first mass filter are subjected to collision induced dissociation. In the collision cell, the ions break into distinct fragments. Under the right settings, the fragmentation pattern is reproducible and unique for each compound.

In the second mass filter, only product ions (fragments) characteristic of the analytes of interest are selected based on their m/z, these are the ions that are detected. In the work presented in this thesis, the product ions m/z 102.1, 88 and 76 are used for BMAA identification.

LC-MS/MS has four criteria for analyte detection: retention time, m/z of the precursor ion, m/z of the product ions and the ratio between the product ions. Compounds that have a precursor with the same m/z and that (partly) give similar product ions as BMAA (green triangle, e.g. DAB) can interfere with the BMAA signal. These compounds need to be removed during extraction or separated from BMAA by LC before entering the MS/MS.

Box 1.4. Principle of BMAA detection by ELISA.
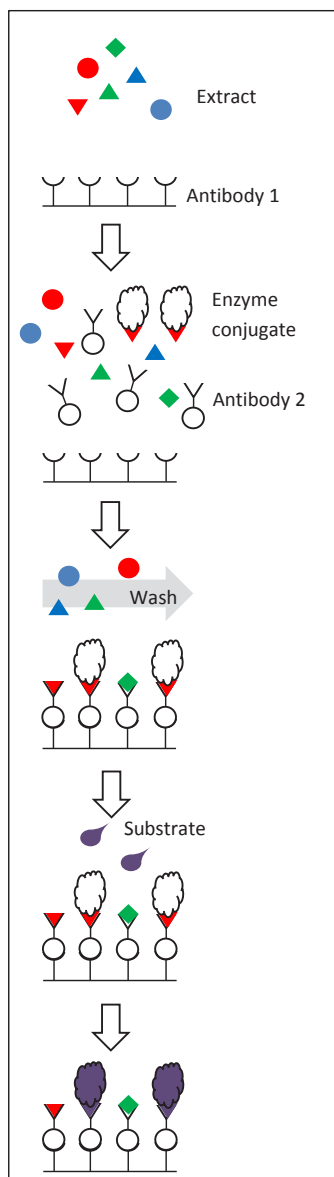
Enzyme Linked Immunosorbent Assay (ELISA) is an *in vitro* test based on binding affinity of the analyte to an antibody.

In the ELISA used in this thesis, a water sample or extract is added to a well of a microtiter plate. The bottom of each well is covered with antibodies (antibody 1 in figure).

After addition of the sample, an enzyme conjugate and a second antibody are added (antibody 2 in figure). Antibody 2 has a binding affinity for BMAA (red triangle) and the enzyme conjugate. Moreover, it can bind to antibody 1. During incubation, BMAA and the enzyme conjugate compete for binding sites on antibody 2, while antibody 2 reacts with antibody 1 and gets fixed to the bottom of the well.

During subsequent wash steps, the dissolved sample components, enzyme conjugates and antibodies that did not react and are therefore not fixed to the well, are removed.

Next, a substrate is added that gives a colour reaction with the enzyme conjugate.

After incubation, the absorbance of each well is read at 450 nm. The intensity of the well colour is inversely related to the amount of BMAA added to the well. ELISAs are generally fast, sensitive and relatively cheap methods. However, there's a risk that other components bind to antibody 2 (green diamond in figure). This so called cross-reactivity can cause false positive signals or analyte overestimation. Therefore, ELISAs are often used for screening purposes, and positive samples are subsequently analysed by more selective techniques.

1.6 OBJECTIVES AND OUTLINE OF THE THESIS

In 2008, when the work for this thesis was started, literature on the occurrence of BMAA in aquatic system was scarce and BMAA was assumed to be present in nearly all cyanobacteria [33-36]. The initial aims of this thesis therefore were to investigate the presence of BMAA in Dutch aquatic systems, to determine its production under different environmental conditions, to determine its toxicity to zooplankton and to determine whether BMAA could accumulate in the aquatic food web. However, shortly after the first experiments for this thesis were started, negative results on BMAA in cyanobacteria were published [76, 78] and a heated discussion started, which mostly focussed on the use of analytical techniques (e.g. [77, 84, 95]). It then seemed more important to find out what had caused the differences in published results, and to find out which data on the presence of BMAA in aquatic systems were reliable. The focus of this thesis therefore changed, and as outlined below, most work was devoted to developing and selecting suitable analytical methods for BMAA analysis and reviewing BMAA literature in search for reliable data on the presence of BMAA in aquatic systems.

This thesis starts with a screening of Dutch urban waters for the presence of BMAA and DAB by LC-MS/MS without derivatisation. As shown in Chapter 2, BMAA was found in nine out of the 21 cyanobacteria dominated samples tested, at a maximum concentration of 42 µg/g DW. The objective of the work presented in Chapter 3 was to find the causes of the discrepancy in published results. Three analytical methods were developed, one based on optical detection (LC-FLD) using AQC derivatisation, one LC-MS/MS method using AQC derivatisation and one underivatised LC-MS/MS method. It was shown that the LC-FLD method risks misidentification of BMAA and might therefore overestimate BMAA concentrations in cyanobacterial samples. In Chapter 4, a commercially available ELISA kit for the analysis of BMAA was tested and it was shown to be highly cross-reactive and therefore unsuitable for the analysis of BMAA in water samples. Given the knowledge that results obtained by optical detection techniques and ELISA might be unreliable, a literature review described in Chapter 5 was performed. The aim of this review was to find out what we really know about the presence of BMAA in aquatic systems. From all reports on BMAA detection in aquatic systems, the studies that used appropriate analytical techniques and that properly reported their work were selected. In the studies that met these criteria, BMAA was either found in phytoplankton and higher aquatic organisms in concentrations in the ng/g DW to low µg/g DW range, or was not detected in studies that used analytical methods with detection limits in the µg/g DW range. As there now was sufficient evidence to assume that BMAA could be present in phytoplankton, the effects of BMAA exposure on the grazer *D. magna* were determined. In Chapter 6, trans generational effects of BMAA exposure on *D. magna* were investigated and it was found that BMAA was transferred from mother to offspring, and that the experimental animals did not adapt to BMAA exposure. Instead, two generations exposure led to higher brood mortality and lower neonate weight

than single generation exposure. The final work for this thesis consisted of organising and instructing a CYANOCOST initiated workshop on BMAA analysis. During this workshop, analytical methods for BMAA determination in a variety of matrices were discussed and tested, the results of which are presented in Chapter 7. Chapter 8 describes a standard operating procedure for LC-MS/MS analysis of underivatised BMAA in cyanobacteria and in Chapter 9, the work performed in this thesis is integrated and discussed.

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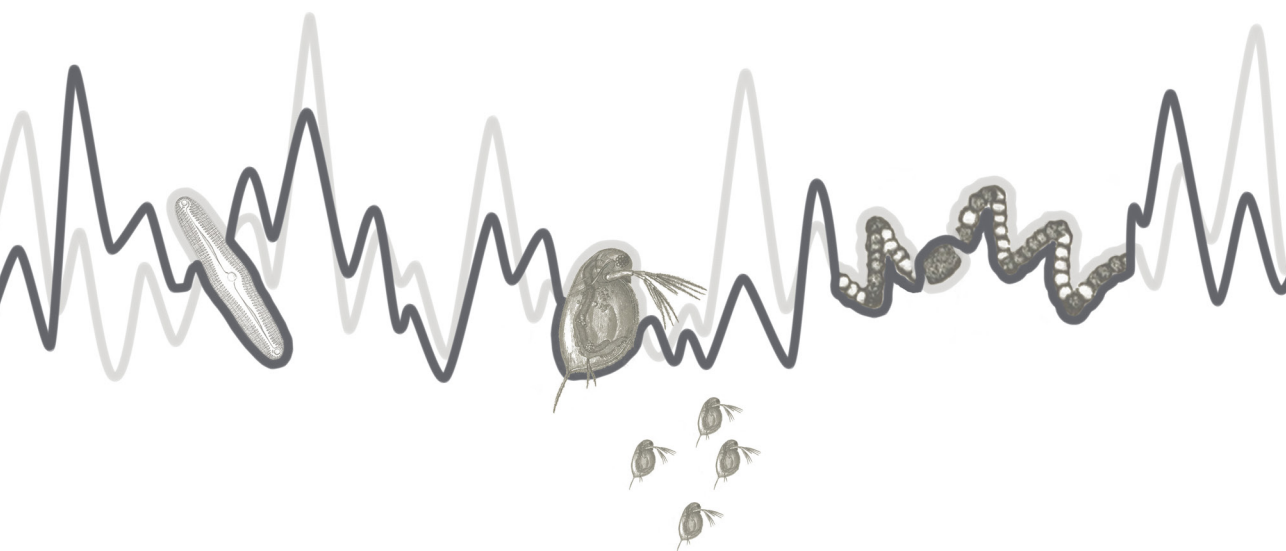
"Aaah Aaah Aaah
There's something in the water, something in the water"

Brooke Fraser, Something in the water
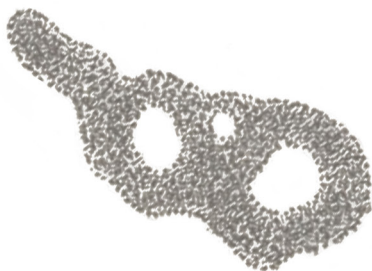
CHAPTER 2

PRESENCE OF BMAA IN DUTCH URBAN WATERS

ABSTRACT

We aimed to determine concentrations of the neurotoxic amino acids β-*N*-methylamino-L-alanine (BMAA) and α-,γ-diaminobutyric acid (DAB) in mixed species scum material from Dutch urban waters that suffer from cyanobacterial blooms. BMAA and DAB were analysed in scum material without derivatisation by LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry) using hydrophilic interaction liquid chromatography (HILIC). Our method showed high selectivity, good recovery of added compounds after sample extraction (86% for BMAA and 85% for DAB), acceptable recovery after sample hydrolysation (70% for BMAA and 56% for DAB) and acceptable precision. BMAA and DAB could be detected at an injected amount of 0.34 pmol. Free BMAA was detected in 9 of the 21 sampled locations at a maximum concentration of 42 µg/g DW. Free DAB was detected in 1 location at 4 µg/g DW. No protein-associated forms were detected. This study is the first to detect underivatised BMAA in cyanobacterial scum material using LC-MS/MS. Ubiquity of BMAA in cyanobacteria scums of Dutch urban waters could not be confirmed, where BMAA and DAB concentrations were relatively low; however, their co-occurrence with other cyanobacterial neurotoxins might pose a serious health risk, including chronic effects from low-level doses.

2.1 INTRODUCTION

The non-protein amino acid β-*N*-methylamino-L-alanine (BMAA) is a neurotoxin [62, 64] that was identified first in cycad seeds [31, 46]. BMAA has been linked to the high incidence of the neurodegenerative disease amyotrophic lateral sclerosis/Parkinson dementia complex (ALS/PDC) among the indigenous Chamorro people on the islands of Guam and Rota in the 1950s [43, 70, 71]. In 2003, Cox and co-workers reported that BMAA was produced by a symbiotic cyanobacterium (*Nostoc* sp.) within specialized roots of the cycad *Cycas micronesica* [32]. Subsequent analysis of cyanobacterial strains from different origins revealed that nearly all tested cyanobacteria contained BMAA [33, 36].

Cyanobacteria are ubiquitous, often flourishing in surface waters that have been over-enriched with nutrients, where they may build massive blooms and form surface scums. Hence, there is a potential for widespread human exposure to BMAA [33]. This seems to be confirmed by analysis of scum material from surface waters in the UK that are used for drinking water and recreation [35].

The presence of BMAA in cyanobacteria has been demonstrated by detection of pre-column derivatised BMAA with at least five different analytical techniques, i.e. HPLC-FLD, UPLC-UV, LC-MS, LC-MS/MS and GC-MS [32-36, 107], and with an amino acid analyser, which uses post-column derivatisation [34]. Despite the multitude of analytical confirmations of BMAA in cyanobacteria, the validity of these data has recently been questioned because some of the methods used are not very specific for BMAA [76]. One study has tried to overcome this drawback by using multiple techniques on one sample [34]. In that study, low levels of BMAA are found in the tested cyanobacterial strain.

During (post- or pre-column) derivatisation, compounds other than BMAA will be derivatised as well, producing derivatiseds with similar optical properties as the BMAA derivatised. When applying optical detection techniques, the use of a confirmatory method such as LC-MS/MS is therefore very important. Our paper extends current BMAA research by including a LC-MS/MS method for the detection of underivatised BMAA in cyanobacteria.

Interestingly, in a recent study using underivatised LC-MS/MS analysis, BMAA was not detected in cyanobacterial samples, but its isomer, the neurotoxic amino acid α-,γ-diaminobutyric acid (DAB) was [76]. DAB had previously been found in association with BMAA in cycad tissues [42]. DAB also occurs in several species of the legume *Lathyrus* [108], in polymixin antibiotics [109] and the cell walls of some plant pathogenic bacteria [110].

Hence, most studies point to a potential exposure to cyanobacterial neurotoxic amino acids (BMAA and/or DAB) of people recreating in surface waters with cyanobacterial blooms. Therefore, the objective of this study was to determine concentrations of BMAA and DAB in

scum material from urban waters in The Netherlands that suffer from cyanobacterial blooms. Analysis was performed on a LC-MS/MS without derivatisation to ensure high selectivity.

2.2 MATERIAL AND METHODS

2.2.1 Sampling

Samples were taken from 21 urban waters with cyanobacterial blooms in the Netherlands. Sampling took place in 2006 and 2007, locations were visited once. Cyanobacterial scums were collected and dominant species were qualitatively determined by light microscopy. Subsequently, samples were lyophilized and stored up to 1.5 year at -20°C until further analysis.

2.2.2 Sample preparation

Sample preparation for LC-MS/MS analysis was based on Murch *et al.*, 2004 [70], BMAA and DAB were analysed as free fractions and protein-associated fractions. The free fractions of 0.5 mg sample were extracted with 150 µl 0.1 M trichloroacetic acid (TCA) for 48 hours at 4 °C. After incubation, the samples were vortexed and centrifuged for 5 minutes at 15,800 × g. The supernatant was transferred to an Eppendorf tube and 150 µl 0.1 M TCA was added to the pellet. The sample was vortexed again and centrifuged. The supernatant was transferred to the Eppendorf tube, where both supernatant fractions were mixed. The supernatant was used for LC-MS/MS analysis of the free fractions. To obtain the protein-associated fractions, the pellet was dissolved in 200 µl hot 6N HCl, flushed with nitrogen gas for 1 minute and brought into vacuum. The pellet was hydrolysed for 20 hours at 112 °C. After hydrolysation, the samples were lyophilized for 24 hours. Finally, 200 µl 20 mM HCl was added to the lyophilized samples after which the protein-associated fractions could be analysed by LC-MS/MS. Samples were either analysed directly after preparation or stored at -20 °C (up to one year) until analysis.

2.2.3 LC-MS/MS analysis

LC-MS/MS analysis was based on the procedure described by Rosén and Hellenäs [76]. The instruments used were an Agilent 1200 LC and an Agilent G6410A QQQ. Chromatography was performed on a 2.1*150 mm, 5 µm diameter ZIC-HILIC column (SeQuant, Sweden) with a Direct-Connect Filter (Grace Alltech). The mobile phase consisted of 65% acetonitrile, 35% water and 0.1% (v/v) formic acid. HPLC flow was 0.4 ml/min, injection volume 5 µl, column temperature 40 °C. The LC-MS/MS was operated in positive mode with an ESI source. Nitrogen was used as the drying and the collision gas. Both quadrupoles were operated in unit mode, fragmentor voltage was 50 V. In MRM mode, quadrupole 1 selected ions with a mass-to-charge ratio (m/z) of 119 and quadrupole 2 recorded the abundance of the following product ions: m/z 101 and 102 at 4 V collision energy, m/z 74, 76 and 88 at 8 V, m/z 73 at 10 V, m/z 56 at 12 V and m/z 44 at 24 V. Calibration standards of BMAA (BMAA Hydrochloride, Sigma-Aldrich) and DAB (DAB Dihydrochloride, Sigma-Aldrich) were prepared on the day of

analysis. Calibration standards were dissolved in 0.1 M TCA for analysis of the free fractions and in 20 mM HCl for analysis of the protein-associated fractions.

We tested recovery by adding known amounts of BMAA and DAB (0.046 µg BMAA and 0.042 µg DAB, dissolved in 20 µl water) to either a blank sample (no matrix) or to 0.5 mg cyanobacterial sample that did not contain detectable amounts of both analytes. BMAA and DAB were added at one of the four stages of sample preparation: 1) before TCA extraction, 2) after TCA extraction, 3) before hydrolysis or 4) after hydrolysis (Figure 2.1). Sample preparation and LC-MS/MS analysis were performed as described above. Each addition was replicated 5 times. Recovery (R) was calculated as R = 100 × (m/s), where m is the measured amount and s is the spiked amount. The measured amount was calculated against a standard curve.
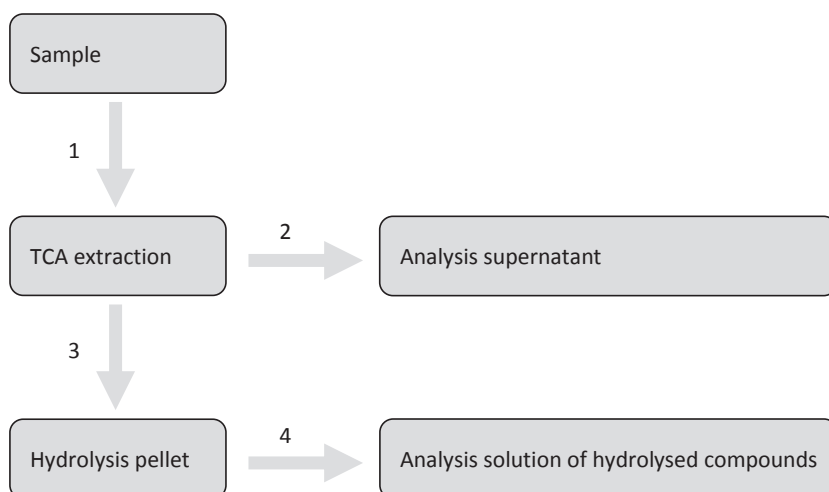
Figure 2.1. Design of the recovery experiment. Numbers in circles indicate moments of BMAA and DAB addition during sample preparation: 1) to sample before TCA extraction, 2) to supernatant after TCA extraction, 3) to pellet before hydrolysis and 4) to solution of hydrolysed compounds.

2.3 RESULTS

BMAA and DAB were both detected at m/z 119 in a MS2 scan. BMAA and DAB were separated by retention time; the retention time of BMAA was shorter than that of DAB (Figure 2.2). Retention times for both compounds differed with the solvent used, retention times were shorter with TCA than with HCl. Furthermore, in both solvents the retention time of cyanobacterial samples was shorter than the retention time of calibration standards. This shift in retention time coincided with an increase in column pressure. In cyanobacterial samples, more peaks of the ion m/z 119 were present in the chromatogram, but because these peaks had a very short retention time, they did not interfere with the peaks of BMAA and DAB.
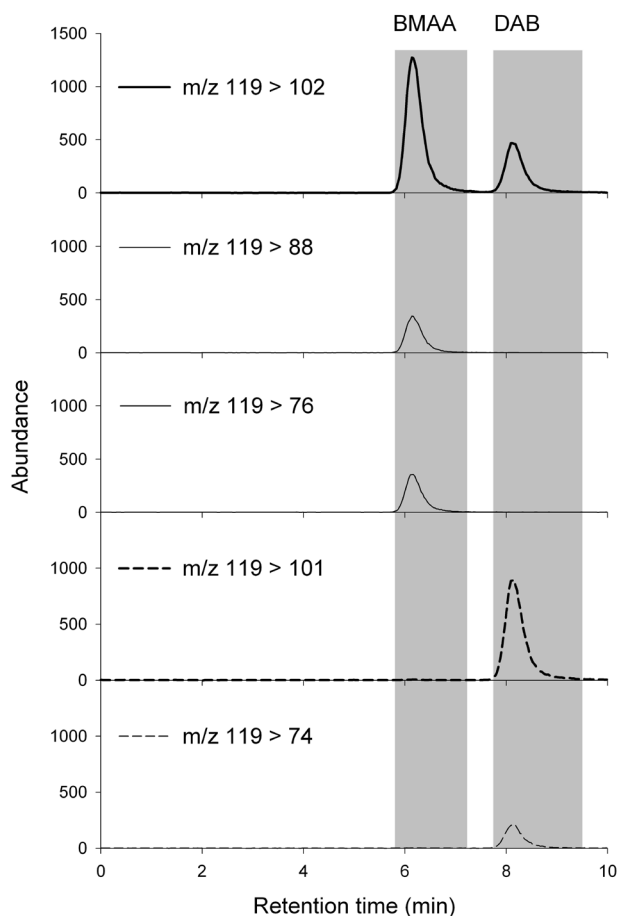
Figure 2.2. LC-MS/MS chromatogram of a calibration standard containing 772 µg/l BMAA and 420 µg/l DAB, dissolved in 20 mM HCl. Solid lines indicate ions used for BMAA determination, dashed lines indicate ions for DAB determination. Bold lines indicate quantifier ions, normal lines indicate qualifier ions. Grey bars indicate retention times of BMAA and DAB.

For BMAA, *m/z* 102 was chosen as quantifier ion because it was the most abundant product ion. The ion *m/z* 102 is not unique to BMAA, it also occurs in the DAB signal (Figure 2.2). Product ions *m/z* 88 and *m/z* 76 were chosen as qualifier ions for two reasons. First, both ions had a relatively high abundance in the BMAA signal and were not detected in the DAB signal. Second, the background signal of these ions was low, so their ratio to *m/z* 102 was constant even at low concentrations of BMAA (8 µg/l). The relative abundance of the qualifier ions to *m/z* 102 was 26% for *m/z* 88 and 27% for *m/z* 76.

For DAB, *m/z* 101 was chosen as quantifier ion because it was the most abundant product ion. Product ion *m/z* 74 was chosen as qualifier ion for two reasons. First, it had a relatively high abundance in the DAB signal and was not detected in the BMAA signal. Second, it gave

the most constant signal at low levels of DAB (8 µg/l). The relative abundance of *m/z* 74 to *m/z* 101 was 23%.

The detection limit of the method is defined as the level at which the ratio quantifier ion/ qualifier ion(s) was within a 20% relative range of the expected value. This criterion was mostly met at a signal to noise level for the quantifier ion of 3. The detection limit of this method for both BMAA and DAB is 0.34 pmol injected on column. When 0.5 mg cyanobacterial sample is used for preparation, this leads to detection limits of approximately 4 µg/g DW for BMAA and DAB in the free fractions, 6 µg/g DW for BMAA in the protein-associated fraction and 8 µg/g DW for DAB in the protein-associated fraction. The detection limits for the protein-associated fractions are already corrected for loss during sample preparation and analysis (see below).

In the recovery experiment nearly all of the added BMAA and DAB was recovered in the blank samples (Table 2.1). In spiked cyanobacterial samples recovery of the added compounds was lower. Approximately 30% of BMAA was not recovered after hydrolysis. For DAB, losses occurred during hydrolysis and analysis of the protein-associated fraction. 44% of the DAB that was added before hydrolysis was not recovered after measurement. For most treatments, the variation between replicates was relatively large with a SD varying between 2 and 12 (Table 2.1).

Table 2.1. Recovery (R, %) of BMAA and DAB added to blank and cyanobacterial samples during sample preparation: 1) to sample before TCA extraction, 2) to supernatant after TCA extraction, 3) to pellet before hydrolysis and 4) to solution of hydrolysed compounds. Amounts of added compounds are 0.046 µg BMAA and 0.042 µg DAB, dissolved in 20 µl water.

Blank samples					
		R added BMAA (%)		R added DAB (%)	
Moment of addition	n	Average	SD	Average	SD
1) Before TCA extraction	5	103	5	97	5
2) After TCA extraction	5	93	11	89	9
3) Before hydrolysis	3	98	6	100	2
4) After hydrolysis	4	112	9	104	10
Cyanobacterial samples					
		R added BMAA (%)		R added DAB (%)	
Moment of addition	n	Average	SD	Average	SD
1) Before TCA extraction	5	86	9	85	10
2) After TCA extraction	5	92	12	85	10
3) Before hydrolysis	5	70	10	56	9
4) After hydrolysis	4	110	5	77	3

BMAA was detected in cyanobacterial scums in 9 of the 21 locations. DAB was detected in 1 location, where it co-occurred with BMAA (Table 2.2 and Figure 2.3). The highest measured

BMAA concentration was 42 µg/g DW, DAB was found at a concentration of 4 µg/g DW. BMAA and DAB were only detected as free fractions, no BMAA and DAB were detected as protein-associated fractions. In eight of the nine samples in which BMAA was detected, filamentous cyanobacteria (*Aphanizomenon*, *Dolichospermum*, *Planktothrix*) were dominant or subdominant (Table 2.2).

Table 2.2. Concentrations of free BMAA and DAB in cyanobacterial scums in Dutch urban waters and dominant and subdominant cyanobacteria in the scums.

Location	Sampling date	[BMAA][1] µg/g DW	[DAB][1] µg/g DW	Dominant species[2]	Subdominant Species[2]
Nijmegen	18-7-2006	8	n.d.	Do	Pa
Boxtel	24-7-2006	n.d.	n.d.	Wo	Ma, Mf, Do
Bergen-op-Zoom	25-7-2006	4	n.d.	Pa	
Grave	25-7-2006	n.d.	n.d.	Mf	Af
Schijndel	25-7-2006	7	n.d.	Ma	Wo
St-Michielsgestel	25-7-2006	n.d.	n.d.	Wo	Ma, Do
Etten Leur	27-7-2006	6	n.d.	Ma	Pa, Do
Budel	1-8-2006	42	n.d.	Do	
Deurne	1-8-2006	n.d.	n.d.	Ma	
Eindhoven	1-8-2006	n.d.	n.d.	Ma	
Wageningen	4-8-2006	n.d.	n.d.	Ma	Mf
St-Oedenrode 1	1-9-2006	37	n.d.	Af	Pa, Do, Ma
Berkel-Enschot	18-10-2006	n.d.	n.d.	Gl	
St-Oedenrode 2	20-10-2006	4	n.d.	Pa	Wo, Ma
Tilburg 1	21-12-2006	6	4	Pa	Wo
Heikant	12-4-2007	n.d.	n.d.	Ag	
Arnhem	9-5-2007	n.d.	n.d.	Wo	Ma, Mf
Beek en Donk	1-8-2007	4	n.d.	Ma	Do
Tilburg 2	8-8-2007	n.d.	n.d.	Wo	Ma
Almere	28-8-2007	n.d.	n.d.	Ma	
Huizen	28-8-2007	n.d.	n.d.	Ma	

[1] n.d. = not detected; [2] Af = *Aphanizomenon flos-aquae*; Ag = *Aphanizomenon gracile*; Do = *Dolichospermum* sp.; Gl = *Gloeotrichia*; Ma = *Microcystis aeruginosa*; Mf = *Microcystis flos-aquae*; Pa = *Planktothrix agardhii*; Wo = *Woronichinia naegeliana*.
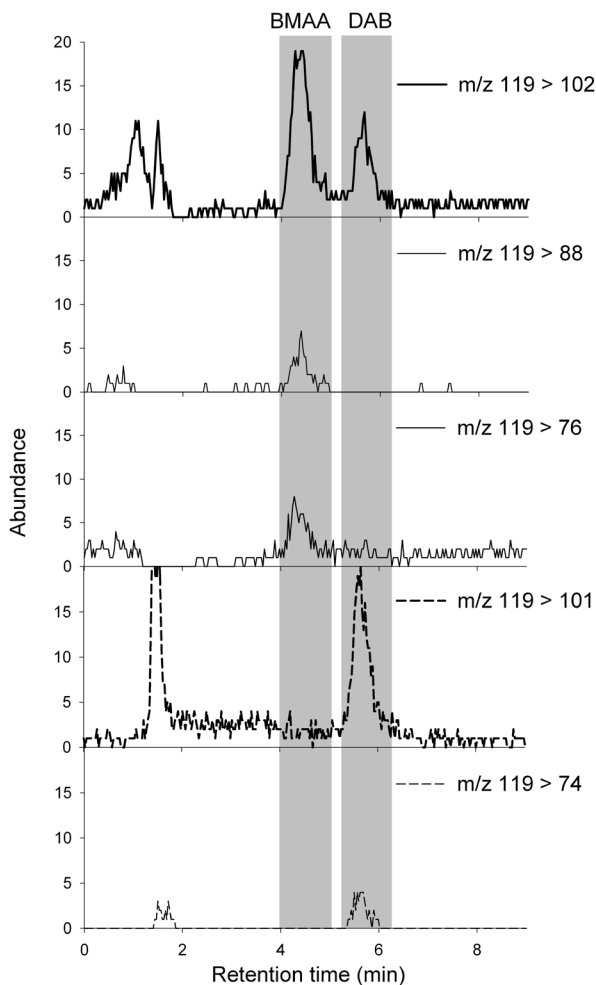
Figure 2.3. LC-MS/MS chromatogram of the free fraction of sample 'Tilburg 1' in 0.1 M TCA. Solid lines indicate ions used for BMAA determination; dashed lines indicate ions for DAB determination. Bold lines indicate quantifier ions, normal lines indicate qualifier ions. Grey bars indicate retention times of BMAA and DAB.

2.4 DISCUSSION

The results of this study clearly show that it is possible to detect and quantify underivatised BMAA and DAB in cyanobacteria dominated samples using LC-MS/MS. All three criteria for identification of the analytes [34] were met as both compounds could be identified by 1) the retention time, 2) the mass-to-charge ratio and 3) the ratio of product ions. Our results do not support the few recent studies that could not detect BMAA in cyanobacteria using a method without derivatisation [76, 78, 111], but also differ from those that used derivatisation techniques and found BMAA in nearly all cyanobacterial samples [33-36,

107]. The latter studies revealed BMAA levels ranging from a few to 276 μg/g DW in bloom samples and up to a few thousand μg/g DW in laboratory cultures in both free and protein-associated fractions. In the present study, measured BMAA levels in mixed species scum samples were maximally a few dozen μg/g DW and BMAA was only detected in the free fraction.

This difference in concentration ranges could be caused by an underestimation of the free BMAA levels in this study. Scum samples were stored at -20°C for a year after preparation, but were thawed for a few days during this storage period. Stability of BMAA in the samples during storage has not been tested. However, it can also not be ruled out that other authors have overestimated BMAA levels in cyanobacteria due to the possible interference of other compounds during analysis [76].

It is unclear why in this study no protein-associated BMAA and DAB were detected in mixed species cyanobacterial scums from the Netherlands. Studies that use derivatisation have reported protein-associated BMAA in nearly every cyanobacterial sample [33-36, 107], in cycads [70, 112] and flying foxes [113]. DAB has been detected in hydrolysed cyanobacterial samples without derivatisation [76]. During this study, the original BMAA and DAB calibration standards in 20 mM HCl had remained stable. To check whether the lack of detection of protein-associated BMAA could have been caused by instability during storage, two laboratory strains and two cyanobacterial scums were hydrolysed and measured directly without storage. No BMAA or DAB were detected in these samples either. Another explanation for the absence of BMAA and DAB in the hydrolysed fractions could, therefore, be that the compounds were not released during hydrolysis in a free state, but reacted with other compounds within the matrix or solvent and could not be detected at m/z 119. For instance, complexation of metal ions with BMAA has been reported [87]. This process is unlikely to cause the total absence of signal in the hydrolysed fractions however, as most of the spiked BMAA and DAB could be recovered. BMAA and DAB might also have been incorporated in smaller peptides that were extracted with TCA but were not precipitated during centrifuging. This sort of association of non-protein amino acids with peptides is not unusual in cyanobacteria. The cyanobacterial toxins microcystins for example are heptapeptides that contain three unusual amino acids [114, 115]. Subsequent research will therefore include hydrolysation of the extraction solution, which will reveal whether BMAA and DAB are present in such small, extractable peptides. Finally, it is also possible that no protein-associated BMAA or DAB were present in concentrations exceeding the detection limits of 6 μg/g DW BMAA and 8 μg/g DW DAB.

This is the first study that detected BMAA in cyanobacteria without derivatisation; in contrast, a few recent studies could not detect BMAA in cyanobacteria using a method without derivatisation [76, 78, 111]. Possible reasons for the unsuccessful detection of

BMAA in those studies are a low sensitivity [111] and only a small number of samples tested [78]. Another recent LC-MS/MS study [76], however, reported a relatively high sensitivity and a wide range of analysed cyanobacterial samples. Differences between the results of that study and our study are difficult to explain. This discrepancy and other unexplained issues on levels and analysis of BMAA in cyanobacteria urge a comparative study where cyanobacterial samples are tested by different laboratories using different methods.

From this study it appears that BMAA and to a lesser extent DAB are present in relatively low concentrations in Dutch urban waters which suffer from cyanobacterial blooms. Health risks involved in the presence of these compounds are unclear because exposure is not quantified and dose-effect relations are not yet available. However, elevated BMAA levels have not only been detected in brain biopsies of Chamorros that died from ALS/PDC, but also in brain tissue of Northern Americans that died from Alzheimer's disease and ALS, while no to very low concentrations of BMAA were detected in patients who had died of a non-neurodegenerative cause [71, 116]. Those results point towards a potential role of BMAA in human neurodegenerative disease outside Guam, in which cyanobacteria might be important players [39].

It is known that low concentrations of BMAA can enhance the effect of other neurotoxins at cellular level [64]. Also, BMAA co-occurs with the cyanobacterial neurotoxins DAB, anatoxin and saxitoxin [35]. We, therefore, advise further research be carried out on the synergistic effects of BMAA and other cyanobacterial neurotoxins.

ACKNOWLEDGEMENTS
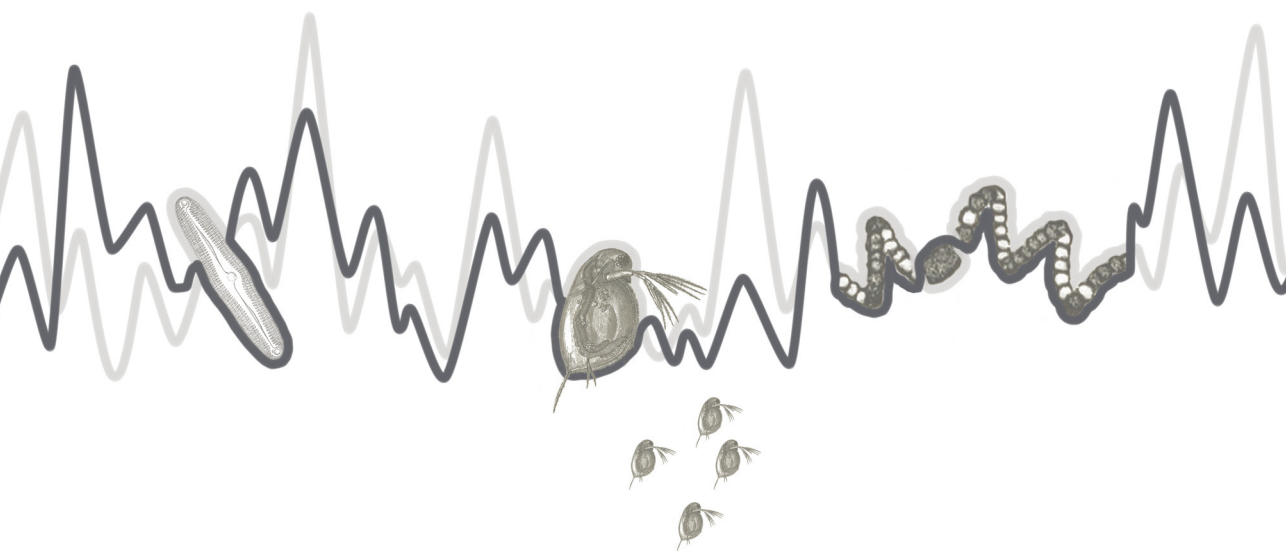
"Since different investigators using different methods have analysed different materials, it is difficult to determine the comparability of differing results"

Banack *et al*, 2010 Toxicon

CHAPTER 3

Comparing three analytical
methods for BMAA quantification

ABSTRACT

The cyanobacterial neurotoxin β-*N*-methylamino-L-alanine (BMAA) has been considered a serious health threat because of its putative role in multiple neurodegenerative diseases. First reports on BMAA concentrations in cyanobacteria were alarming: nearly all cyanobacteria were assumed to contain high BMAA concentrations, implying ubiquitous exposure. Recent studies however question this presence of high BMAA concentrations in cyanobacteria. To assess the real risk of BMAA to human health, this discrepancy must be resolved. We therefore tested whether the differences found could be caused by the analytical methods used in different studies.

Eight cyanobacterial samples and two control samples were analysed by three commonly used methods: HPLC-FLD analysis and LC-MS/MS analysis of both derivatised and underivatised samples. In line with published results, HPLC-FLD detected relatively high BMAA concentrations in some cyanobacterial samples, while both LC-MS/MS methods only detected BMAA in the positive control (cycad seed sarcotesta). Because we could eliminate the use of different samples and treatments as causal factors, we demonstrate that the observed differences were caused by the analytical methods.

We conclude that HPLC-FLD overestimated BMAA concentrations in some cyanobacterial samples due to its low selectivity and propose that BMAA might be present in (some) cyanobacteria, but in the low µg/g or ng/g range instead of the high µg/g range as sometimes reported before. We therefore recommend to only use selective and sensitive analytical methods like LC-MS/MS for BMAA analysis. Although possibly present in low concentrations in cyanobacteria, BMAA can still form a health risk. Recent evidence on BMAA accumulation in aquatic food chains suggests human exposure through consumption of fish and shellfish which expectedly exceeds exposure through cyanobacteria.

3.1 INTRODUCTION

The neurotoxic amino acid β-*N*-methylamino-L-alanine (BMAA) has been linked to neurodegenerative diseases as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) [37]. BMAA was first identified in 1967 in the seeds of the cycad *Cycas micronesica* [31] in a survey on the cause of the high incidence of ALS, PD and dementia on Guam [117]. The possible etiological role of BMAA in this neurodegenerative disease was at first disputed [118], but was recently resurrected by the discovery of high concentrations of BMAA in the protein associated fraction of the cycad seeds and its biomagnification in the Guamanian food chain [32, 70, 113, 119]. Furthermore, the finding of BMAA in the brains of people who had died with AD, ALS or PD outside Guam pointed towards a wider occurrence of BMAA [69, 71]. The detection of BMAA in the cyanobacterium *Nostoc sp.* that lives in symbiosis with the cycads [32] prompted the screening of cyanobacteria from all over the world for BMAA [33, 35, 36, 76, 77, 83, 94, 102, 107, 111, 120-122].

First reports on BMAA concentrations in cyanobacteria were alarming: high concentrations of BMAA were detected in nearly all tested free living laboratory strains [33], field isolates [36], field samples [35] and symbiotic species [33] (Table 3.1). In contrast, all but one [121] later studies could not reproduce these first results; BMAA was either not detected in cyanobacteria (e.g. [76, 77]), detected in some, yet not all, samples [102] or detected in all samples, but at very low concentrations [83]. The suggestion that BMAA might have been confused with its structural isomer α,γ-diaminobutyric acid (DAB) in the early studies [77] could be refuted [84].

Table 3.1. Overview of studies that analysed more than eight samples of free living cyanobacteria for BMAA.

Tested samples	Fraction samples positive for BMAA	BMAA concentration in positive samples [a]	Analytical quantification method	Derivatisation method	Reference
(n)	(-)	(μg/g DW)			
8	1.00	402 (190-1110)	CE	None	[121]
30	0.97	968 (10-6721)	HPLC-FLD	AQC [b]	[33]
12	1.00	103 (8-287)	HPLC-FLD	AQC	[35]
27	0.96	129 (0.1-2757)	GC-MS	EZ:faast	[36]
21	1.00	$6.6*10^{-3}$ ($1*10^{-3}$-$15*10^{-3}$)	LC-MS/MS	AQC	[83]
20	0.95	1.35 (0.05-10.7)	LC-MS	EZ:faast	[122]
21	0.42	13 (4-42)	LC-MS/MS	None	[102]
36	0.00	-	LC-MS/MS	None	[76]
30	0.00	-	LC-MS/MS	None	[77]

[a] BMAA concentration is the sum of the free and protein associated concentrations. Values are averages, followed by minimum and maximum concentrations between brackets. [b] AQC: 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate

Nonetheless there is still little consensus on BMAA concentrations in cyanobacteria. Cyanobacteria are ubiquitous and multiple routes of human exposure to cyanobacteria and their toxins exist [123]. It is therefore very important for human risk assessment to find the cause of the discrepancy in published results on BMAA concentrations in cyanobacteria.

Several factors underlie the different studies. Researchers have used different samples, different sample treatments and different analytical methods [84]. However, the differences in results seem to be related to the analytical method used. High BMAA concentrations and high percentages of positives samples were found only in those studies that had used high performance liquid chromatography with fluorescence detection (HPLC-FLD), gas chromatography with mass spectrometry detection (GC-MS) or capillary electrophoresis (CE) for quantification (Table 3.1). On the other hand, studies that had used high performance liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) for quantification either did not detect BMAA, or reported lower BMAA concentrations (Table 3.1). Therefore, we hypothesized that different analytical methods for the determination of cyanobacterial BMAA deviate in their results. To test the hypothesis, we analysed a set of cyanobacterial and control samples with three analytical methods: HPLC-FLD, LC-MS/MS of derivatised samples and LC-MS/MS of underivatised samples. The observed differences in our study were comparable to the observed differences in literature and were caused by overestimation of BMAA concentrations by HPLC-FLD.

3.2 RESULTS

3.2.1 Method validation

Before sample analysis, all three methods were validated. Results of method validation are shown in Table 3.2. LC-MS/MS analysis of samples was performed with deuterium labelled BMAA (D$_3$BMAA) as an internal standard. However, the validation of both LC-MS/MS methods was performed without correction for the internal standard to make comparison with the HPLC-FLD method possible. HPLC-FLD response was linear up to a concentration of 1000 µg/l, while both LC-MS/MS responses were linear up to 500 µg/l (Table 3.2). When corrected for the response of D$_3$BMAA however, the LC-MS/MS methods showed a broader range of linearity (Figure A3.1). For all three methods, the fit of the regression line was good ($r^2 > 0.999$).

Detection and quantification limits of calibration standards were within the same range for all three methods. For the LC-MS/MS methods, the limit of detection (LOD) often equalled the limit of quantification (LOQ). This is possible because for these methods, LOD is defined as the lowest concentration where the signal-to-noise (S/N) ratio of all product ions is at least 3:1 and the ratio of the qualifier ion(s) to the quantifier ion is within a 20% relative range. The conditions for the ratio of the qualifier ions to the quantifier ion or for the S/N of the qualifier ions are often only met at a S/N ratio of the quantifier ion of 10:1, in which

Table 3.2. Validation results of the three used methods, both LC-MS/MS methods are validated without correction for the internal standard D$_3$BMAA.

		HPLC-FLD	LC-MS/MS derivatised	LC-MS/MS underivatised
Linearity [a]				
Lowest concentration	µg/l	15	5	7.5
Highest concentration	µg/l	1000	500	500
Number of concentrations in tested range	-	6	8	9
r^2	-	0.999	0.999	0.999
Detection and quantification limits				
LOD calibration standard	fmole/injection	68	85	106
LOQ calibration standard	fmole/injection	102	85	317
LOD sample extract	µg/g	*	1.0	0.4
LOD sample hydrolysed	µg/g	40	10.0	1.6
LOQ sample extract	µg/g	*	1.0	0.4
LOQ sample hydrolysed	µg/g	120	10.0	1.6
Precision				
Intraday precision (n=6), response	Relative SD (%)	2.8	3.0	0.7
Intraday precision (n=6), RT	Relative SD (%)	0.0	0.1	0.1
Interday precision (n=12), response	Relative SD (%)	4.6	5.0	1.9
Interday precision (n=12), RT	Relative SD (%)	0.1	0.0	0.2
Inter workup (n=12) extract, response	Relative SD (%)	8.6	6.7	7.1
Inter workup (n=12) extract, RT	Relative SD (%)	0.1	0.0	0.4
Inter workup (n=12) hydrolysed, response	Relative SD (%)	17.0	10.6 [b]	6.1
Inter workup (n=12) hydrolysed, RT	Relative SD (%)	0.1	0.1 [b]	0.1

LOD: limit of detection, LOQ: limit of quantification, RT: retention time, [a] each concentration is injected in triplicate, [b] n=11, * not determined (see text)

case the criteria for LOQ are also met. Chromatograms of LODs in samples are shown in Figure A3.2. Detection limits in samples are higher for both derivatised methods than for underivatised LC-MS/MS analysis. This is due to the dilution during derivatisation (both extracts and hydrolysed samples) and the extra dilution that is needed in the hydrolysed samples to ensure effective derivatisation [124, 125]. A single LOD or LOQ of free BMAA in samples could not be determined for the HPLC-FLD method. The extracts of cyanobacterial samples showed many low peaks around the retention time of BMAA, which made a good estimation of the position of the baseline difficult. For each sample, the pattern of these peaks was different, so no universal LOD or LOQ could be derived. This problem did not occur in the hydrolysed samples, these chromatograms all showed fewer but higher peaks, with a better definable baseline for BMAA. Because baseline variation was higher for HPLC-FLD analysis than for LC-MS/MS analysis of derivatised samples, detection and quantification limits of the latter method in samples were lower. Underivatised LC-MS/MS was the most sensitive method for analysis of both extracted and hydrolysed samples.

Interday and intraday precision was good for all three methods; underivatised LC-MS/MS analysis was most precise. Inter workup of hydrolysed samples analysed by HPLC-FLD

showed an unexplainable high variation. Retention times in HPLC-FLD analysis were sensitive to variations in the buffer solution. This resulted in retention time differences between runs of maximum 0.3 min. Within runs, retention times were stable (Table 3.2).

Of the samples that were spiked before extraction, between 83.6 and 86.8% of the expected signal was recovered. Samples that were spiked before hydrolysis showed a lower recovery: between 46.7 (HPLC-FLD) and 69.3% (underivatised LC-MS/MS, Table 3.3).

Table 3.3. Recovery (%) of extraction and hydrolysis, analysed by the three different methods.

	Extraction			Hydrolysis		
	average	SD	n	average	SD	n
HPLC-FLD	86.8	10.1	12	46.7	8.5	12
LC-MS/MS derivatised [a]	83.6	5.5	12	68.6	6.8	11
LC-MS/MS underivatised [a]	85.5	5.9	12	69.3	4.2	12

[a] recovery is calculated for D_3BMAA

All three methods separated BMAA from its isomer DAB (Figure 3.1).
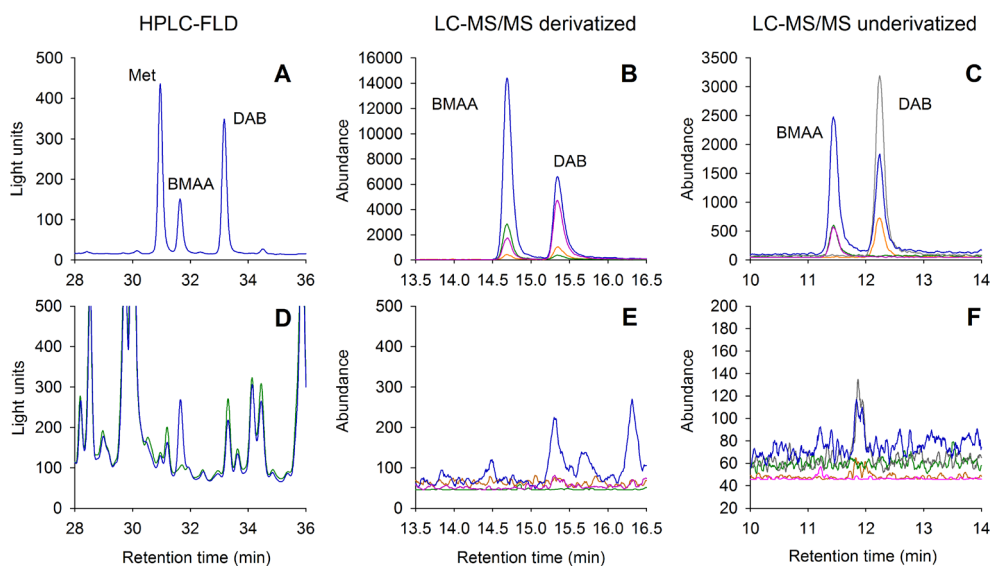
Figure 3.1. Chromatograms of the three analytical methods showing calibration standards and an extracted cyanobacterial sample. Panels A- C show calibration standards, panels D- F show the extracted *Dolichospermum* field scum. The green line in panel D represents the unspiked cyanobacterial sample, the blue line indicates the same sample, but spiked with BMAA before extraction. Coloured lines in panels B and E represent the transitions of ions with a mass-to-charge ratio (*m/z*) of 459 to *m/z* 171 (blue), 119 (green), 145 (pink) and 315 (orange). Coloured lines in panels C and F represent the transitions of *m/z* 119.1 to *m/z* 102.1 (blue), 88 (pink), 76 (green), 101 (grey) and 74 (orange). Transitions for D_3BMAA are not shown.

3.2.2 BMAA in samples

Analysis of the same samples by the three methods yielded different results (Table 3.4). Both LC-MS/MS methods only indicated BMAA in the positive control, the sarcotesta of the cycad seed. HPLC-FLD however indicated BMAA not only in the cycad seed, but also in three of the eight cyanobacterial samples.

Table 3.4. Free and total BMAA concentrations (µg/g DW, average and SD, n=3) in control and cyanobacterial samples as analysed by three different methods.

	HPLC-FLD		LC-MS/MS derivatised		LC-MS/MS underivatised	
	Free	Total	Free	Total	Free	Total
Controls						
S. obliquus SAG 276/3a (neg)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cycad seed sarcotesta (pos)	**18.2 (1.4)**	**d.**	**8.8 (3.0)**	**104.9 (4.5)**	**10.7 (2.9)**	**75.0 (10.8)**
Field scums						
Dolichospermum	**21.7 (3.1)**	n.d.	n.d.	n.d.	n.d.	n.d.
P. rubescens	**6.3 (0.8)**	n.d.	n.d.	n.d.	n.d.	n.d.
Aphanizomenon	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Microcystis	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lab strains						
C. raciborskii CS-1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A. flos-aquae CCAP 1401/7	**56.2[a]**	**d.**	n.d.	n.d.	n.d.	n.d.
D. flos-aquae CCAP 1409/2A	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M. aeruginosa NIVA CYA 228/1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: not detected, d.: detected but below limit of quantification, [a] n=2

Of the three methods used, HPLC-FLD has the highest detection limit for BMAA in samples. All BMAA concentrations as determined by HPLC-FLD were therefore far above the detection limit of both LC-MS/MS methods (Tables 3.2 and 3.4). If samples indeed contained BMAA concentrations as high as indicated by HPLC-FLD, both LC-MS/MS methods should have detected BMAA as well.

Free BMAA concentrations in the cycad seed sarcotesta were in the same range for all three methods. Total BMAA was also detected in the cycad seed by all three methods, but was below the LOQ for HPLC-FLD (Table 3.4).

3.3 DISCUSSION

We clearly showed that the determined BMAA concentrations in some cyanobacterial samples varied depending on the analytical method used. The three methods only indicated similar BMAA concentrations in the positive control, the sarcotesta of a cycad seed. Since we have used the same samples and employed identical sample treatments, sample origin and treatment could be eliminated as possible causal factors [84] of the observed differences. The differences in BMAA concentrations can therefore only be attributed to the analytical methods, and are roughly in line with the observed discrepancy in published results (Table 3.1).

HPLC-FLD identified BMAA in three out of eight cyanobacterial samples, while both LC-MS/MS methods did not detect any BMAA in cyanobacteria. These differences are most likely due to the low selectivity of the HPLC-FLD. HPLC-FLD is less selective than both LC-MS/MS methods, it has only two selection criteria: retention time and fluorescence signal. Because BMAA does not have fluorescent properties, derivatisation with the fluorescent AQC was necessary for detection by HPLC-FLD. Both retention time and fluorescence signal are properties of the derivatised of an analyte, instead of the analyte itself and any compound that reacts with AQC gives the same fluorescence signal after derivatisation. AQC reacts with primary and secondary amino groups [106, 126], which means that it reacts with all amino acids and other amino group containing compounds. There are hundreds of naturally occurring amino acids [127], so there is always a chance that a derivatised compound other than BMAA has the same or a similar retention time as the BMAA derivatised. If such a compound is present in a sample, it leads to misidentification and subsequent overestimation of BMAA concentrations in that sample. HPLC-FLD is therefore an uncertain method for amino acid analysis in complex biological matrices that contain non-protein amino acids or other compounds with an amino group [106], especially when the analyte is present in low concentrations.

LC-MS/MS is a more selective method than HPLC-FLD because it has four selection criteria: retention time, mass-to-charge ratio (m/z) of the precursor ion (the charged 'original' molecule), m/z of the product ions after collision induced dissociation and the ratio between the abundance of the product ions. The chance of compound misidentification by LC-MS/MS is therefore much smaller than by HPLC-FLD. In our study, LC-MS/MS peaks were only identified as BMAA when all four criteria were met.

The discrepancy in the results could not be caused by other factors like method sensitivity or difference in sample treatment (derivatisation versus underivatised analysis). In our study, the least sensitive method (HPLC-FLD) gave more positive results than the more sensitive LC-MS/MS methods. Quantification by LC-MS/MS was reliable because we used D_3BMAA as an internal standard in all samples [76], resulting in unbiased estimates of BMAA concentrations in samples, also in low concentrations (Figure A3.3). The observed differences in results can therefore not be explained by differences in method sensitivity. Also derivatisation cannot explain the differences in results, because both derivatised methods (HPLC-FLD and LC-MS/MS analysis of derivatised samples) varied in their outcome. It has been suggested that (underivatised) HILIC LC-MS/MS analysis is less suitable for BMAA detection than LC-MS/MS analysis after derivatisation [84], but our study does not support this hypothesis as underivatised LC-MS/MS analysis was in our case more precise and more sensitive for samples than derivatised LC-MS/MS analysis (Table 3.2). Furthermore, the warning that the signal of methionine methylsulphonium might interfere with that of BMAA in underivatised LC-MS/MS analysis [95] is unnecessary, because this compound has

a different molecular weight than BMAA and its signal will therefore not be picked up in MRM analysis. We conclude that the most likely cause of the differences in our experiment is that HPLC-FLD has misidentified another amino containing compound as BMAA in some samples and consequently has overestimated BMAA concentrations in these samples.

From our study, it cannot be determined which compound has mistakenly been identified as BMAA by HPLC-FLD analysis. While attempts have been made to exclude compounds from being possibly interfering in various methods of BMAA analysis [84, 95], these studies only focus on a few compounds, mostly diamino acids. The possible similarity of the fragmentation pattern of diamino acids with that of BMAA makes these compounds likely candidates for interference with mass spectrometry analyses. The compounds tested in these two studies did not interfere with the BMAA signal in most tested methods, only one compound co-eluted in an UHPLC-UV/MS method [95]. However, the list of possible interfering compounds in HPLC-FLD analysis is much larger and also includes compounds with only one amino group. These two studies can therefore not be used to identify possibly interfering compounds in previously performed HPLC-FLD analyses. Furthermore, different chromatographic conditions can result in different interfering compounds, which means that the compound that has mistakenly been identified in our study, can be another compound than the one that has interfered in other studies.

The average BMAA concentrations found by HPLC-FLD in this study are lower than concentrations found in free living cyanobacteria by previous studies that used HPLC-FLD for quantification [33, 35] (Table 3.1). Furthermore, in our study BMAA was identified in only three of the eight tested cyanobacterial samples, while the other HPLC-FLD studies report presence of BMAA in nearly all tested cyanobacteria. Again, this is most likely due to the differences in chromatographic conditions between the studies. Although presence of BMAA has been confirmed by LC-MS/MS in the early HPLC-FLD based studies, BMAA concentrations determined by these LC-MS/MS analyses have not been reported [33, 35]. It is therefore unknown whether the concentrations found by HPLC-FLD matched the concentrations found by LC-MS/MS in these studies. Also in the only other study, so far, that compared different analytical methods, BMAA concentrations based on LC-MS(/MS) analyses were not reported [34].

Certainly, variations in BMAA concentrations found in different studies may not only be caused by the use of different analytical methods. BMAA concentrations may also differ as a result of the origin or growth conditions of the cyanobacteria, which is the case for most other cyanobacterial toxins [128]. An attempt has been made to determine conditions under which cyanobacteria produce BMAA [99], but much work is still needed to understand BMAA production. Origin and growth conditions cannot explain the incongruity observed in our study because we analysed the same material with different analytical methods. So,

although the high BMAA concentrations measured in the early studies may indeed have resulted from samples that contained high amounts of BMAA, it is more plausible that they are an artefact of the HPLC-FLD method. Our results suggest that BMAA is not present in high concentrations in cyanobacteria. Presence of lower concentrations of BMAA (low µg/g DW or ng/g DW) in (some) cyanobacteria is more likely and could also explain why BMAA is detected by some selective methods with high sensitivity [83, 94] and is not detected [76, 77, 111] or detected only in a number of samples [102] by studies that have a lower sensitivity.

Our study only focused on three analytical methods and can therefore not explain other discrepancies in published BMAA concentrations than those between HPLC-FLD and (derivatised or underivatised) LC-MS/MS analysis. More work is for instance needed to explain the differences in concentrations found in cyanobacterial field isolates from similar regions and grown under similar condition that were analysed by the same group by GC-MS and LC-MS [36, 122]. Also the high concentrations found by CE analysis [121] are interesting, even though the authors of this last manuscript acknowledge that the selectivity of their method is low. In general, comparison of the quantitative results of different studies is hampered by the absence of recovery and validation data in many publications.

Even if BMAA concentrations in cyanobacteria are low, BMAA can still pose a threat to human health. First, BMAA has the ability to accumulate in food chains. In the Baltic sea, BMAA concentrations in zooplankton, shellfish and bottom-dwelling fish species are up to 200 fold higher than in the local cyanobacteria [83]. Moreover, laboratory studies have shown that the zooplankton species *Daphnia magna* is able to take up BMAA from its surrounding medium, thereby bioconcentrating BMAA up to 3800 times [129]. Presence of BMAA in the aquatic food chain means that people are not only exposed to BMAA by direct contact with cyanobacteria, but also through food. Exposure through food may extent over a larger area and a larger period of time than exposure through cyanobacteria. The dose of BMAA obtained through food might therefore exceed the dose obtained directly through cyanobacteria. Second, in addition to its own neurotoxicity, BMAA can also enhance the effect of other neurotoxins [64]. The additive effect of BMAA with other cyanobacterial neurotoxins has not been evaluated yet, but BMAA sometimes occurs simultaneously with the neurotoxins DAB [102], anatoxin-a and saxitoxin [35] and synergistic toxicity cannot on forehand be excluded.

We conclude that in our study HPLC-FLD overestimated BMAA concentrations in some cyanobacterial samples due to its low selectivity. Cyanobacterial BMAA concentrations seem to be overestimated in some previous studies as well and are more likely to be in the low µg/g DW or even in the ng/g DW range than in the high µg/g DW range as sometimes reported. We therefore recommend to only use selective and sensitive analytical methods

like LC-MS/MS for BMAA analysis. Although possibly present in low concentrations in cyanobacteria, presence of BMAA in the aquatic food chain and possible synergistic effects with other cyanobacterial neurotoxins still urge for investigation on the risk of BMAA for human health.

3.4 MATERIALS AND METHODS

Eight cyanobacterial samples (four scum samples from the field and four laboratory strains), a negative control (a green alga) and a positive control (sarcotesta of a cycad seed) were prepared for analysis of free and total BMAA. The sample treatments were the ones that are most often applied: trichloroacetic acid extraction for analysis of free BMAA and acid hydrolysis for total BMAA. All sample treatments were performed in nine fold. Six replicates of each fraction were then derivatised using AQC and analysed by HPLC-FLD (n=3) or LC-MS/MS (n=3). The other three replicates were analysed by LC-MS/MS without derivatisation. Prior to extraction or hydrolysis, deuterium labelled BMAA was added to the samples that were analysed by LC-MS/MS as an internal standard. L-2-aminobutyric acid (AAbA) was added after extraction or hydrolysis to the samples that were analysed by HPLC-FLD and was used as retention time reference. Validation of all methods was based on FDA guidelines [130, 131].

3.4.1 Sample material

The control samples consisted of the green alga *Scenedesmus obliquus* SAG 276/3a (negative control) and the sarcotesta of a *Cycas micronesica* (Hill) seed (positive control). *S. obliquus* was cultured as in [129] and was harvested directly before sample preparation. The cycad seed was kindly provided by Chad Husby, Montgomery Botanical Centre, Miami, US and was stored at -20 °C after picking. The cyanobacterial scum samples were collected in various lakes in The Netherlands in 2008 and 2009 and were dominated by either *Dolichospermum*, *Planktothrix rubescens*, *Aphanizomenon* or *Microcystis*. The cyanobacterial laboratory strains used were *Cylindrospermopsis raciborskii* CS-1, *Aphanizomenon flos-aquae* CCAP 1401/7, *Dolichospermum flos-aquae* CCAP 1409/2A and *Microcystis aeruginosa* NIVA CYA 228/1. The first three strains were grown in batch cultures on a modified WC medium [132] at room temperature at normal daylight and were harvested after a growth period of 20 to 25 days, while *Microcystis* was grown for 15 days at 20°C and in 45 µmol quanta m^{-2}s^{-1} light in a 16:8 h light:dark rhythm. All samples were lyophilized and stored at -20 °C until preparation.

3.4.2 Sample preparation

All lyophilized samples were homogenized and extracted or hydrolysed. 5 mg of sample (0.5 mg for the cycad seed) was extracted for free BMAA at room temperature in the dark for two hours in 300 µl 0.1 N trichloroacetic acid (TCA). After the extraction, the sample was centrifuged and the supernatant was transferred. 300 µl 0.1 N TCA was then again added

to the pellet and after vortexing and centrifugation the supernatant was pooled with the first supernatant and lyophilized. The dried supernatants were derivatised after dissolving them in 500 μl hot 20 mM HCl. Samples were derivatised by adding 60 μl buffer and 20 μl reagent (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), purchased as AccQ®-Tag, Waters) to 20 μl sample [124]. Dried supernatants for underivatised LC-MS/MS analysis were dissolved in 500 μl 65% acetonitrile, 35% Millipore water and 0.1% formic acid (v:v:v).

For total BMAA, 1 mg of lyophilized sample (0.5 mg for the cycad seed) was hydrolysed in an hydrolysis/derivatisation workstation (Eldex), using 6 N HCl liquid hydrolysis for 20 hours at 105 °C in the absence of oxygen. After hydrolysis, samples for derivatised analysis were dissolved in 500 μl hot 20 mM HCl and subsequently diluted ten times in 20mM HCl to obtain a protein concentration below 0.1 g/l ([124, 125], maximum protein content of cyanobacteria was estimated to be 50%). Derivatisation procedure was the same as for the free fraction. Hydrolysed samples for underivatised LC-MS/MS analysis were dissolved in 1 ml 65% acetonitrile, 35% Millipore water and 0.1% formic acid (v:v:v).

Deuterium labelled BMAA (D$_3$BMAA, kindly provided by Johan Rosén, National Food Administration, Uppsala, Sweden and synthesized as in [76]) was added to the samples that were analysed by LC-MS/MS prior to extraction or hydrolysis so the maximum concentration at the moment of analysis was 400 μg/l. L-2-aminobutyric acid (AAbA, Sigma-Aldrich) was added to the samples that were analysed by HPLC-FLD after extraction or hydrolysis at a maximum concentration of 500 μg/l.

3.4.3 Sample analysis

HPLC-FLD analysis was performed on an Agilent 1100 LC-FLD. Compounds were separated on a Nova-Pak C18 3.9×300 mm, 4 μm column (Waters). Eluent A consisted of 140 mM sodium acetate and 5.6 mM triethylamine in Millipore water, adjusted to pH 5.2 with phosphoric acid. Eluent B was acetonitrile and eluent C was Millipore water. The elution program was: 0 min 100% A; 7 min 90% A and 5.2% B; 10-20 min 84% A and 8.3% B; 23 min 75% A and 13% B; 38 min 65% A and 18.2% B; 40 min 40% A and 31.2% B; 42.5-52 min 52% B and 48% C; 55-65 min 100% A. Flow rate was 1 ml/min, injection volume 4 μl and column temperature 37 °C. Excitation wavelength was 250 nm, emission wavelength was 395 nm.

LC-MS/MS analysis of the derivatised samples was performed on an Agilent 1200 LC and an Agilent 6410A QQQ. Compounds were separated on a Zorbax Eclipse AAA 4.6×75 mm, 3.5 μm column (Agilent) with mobile phases acetonitrile with 0.1% formic acid (v:v, eluent A) and Millipore water with 0.1% formic acid (v:v, eluent B). The following gradient was applied: 0 min 1% A; 4 min 2% A; 8 min 5% A; 18 min 10% A; 20-24 min 50% A; 24-38 min 0% A. Flow rate was 1 ml/min, injection volume 10 μl and column temperature 40 °C. The LC-MS/MS was operated in positive mode with an ESI source, fragmentor voltage was 140 V.

Nitrogen was used as the drying and collision gas. Quadrupole 1 was operated in unit mode and quadrupole 2 was operated in widest mode. BMAA was detected by the transitions mass-to-charge ratio (*m/z*) 459 to *m/z* 171 at 32 V collision energy, *m/z* 119 and *m/z* 145 (both 16 V). Ratio of the peak area of qualifier *m/z* 119 to the peak area of quantifier *m/z* 171 was 10%; ratio of the qualifier *m/z* 145 to *m/z* 171 was 14%. DAB was detected by the transitions *m/z* 459 to *m/z* 171 (28 V), *m/z* 145 and *m/z* 315 (both 12 V). Ratio of the qualifier *m/z* 145 to quantifier *m/z* 171 was 83% and ratio of the qualifier *m/z* 315 to *m/z* 171 was 8%. D_3BMAA was detected by the transitions *m/z* 462 to *m/z* 171 (32 V), *m/z* 145 and *m/z* 122 (both 16 V). Ratio of the qualifier *m/z* 145 to quantifier *m/z* 171 was 13% and ratio of the qualifier *m/z* 122 to *m/z* 171 was 23%.

Underivatised samples were analysed on the same LC-MS/MS equipment and with the same mobile phases as the derivatised samples. Compounds were separated on a 2.1×150 mm, 5 µm diameter ZIC-HILIC column (Sequant) with a Direct-Connect Filter (Grace Alltech). Flow rate was 0.4 ml/min, injection volume 5 µl and column temperature 40 °C. The following gradient was applied: 0-2 min 95% A; 4 min 65% A; 8-17 min 55% A; 17-23 min 95% A. Fragmentor voltage was 50 V and both quadrupoles were operated in unit mode. BMAA was detected by the transitions *m/z* 119.1 to *m/z* 102.1 (4 V), *m/z 88* and *m/z* 76 (both 8 V). Ratio of both qualifiers *m/z* 88 and *m/z* 76 to quantifiers *m/z* 102.1 was 21%. DAB was detected by the transitions *m/z* 119.1 to *m/z* 101 (4 V) and *m/z* 74 (8 V). Ratio of the qualifier *m/z* 76 to quantifier *m/z* 101 was 23%. D_3BMAA was detected by the transitions *m/z* 122.1 to *m/z* 105.1 (4 V), *m/z 88* and *m/z* 76 (both 8 V). Ratio of qualifier *m/z* 88 to quantifier *m/z* 105.1 was 22%; ratio of *m/z* 76 to *m/z* 105.1 was 37%.

Calibration standards for the derivatised samples were prepared in 20mM HCl and then derivatised, calibration standards for the underivatised samples were prepared in 65% acetonitrile, 35% Millipore water and 0.1% formic acid (v:v:v). Calibration standards for LC-MS/MS analysis contained BMAA, DAB (DAB dihydrochloride, Sigma-Aldrich) and D_3BMAA. Calibration standards for HPLC-FLD analysis contained BMAA, DAB, methionine (DL-Methionine, Fluka) and AAbA. BMAA concentrations in LC-MS/MS samples were determined by correcting the response of BMAA for the response of D_3BMAA. BMAA concentrations analysed by HPLC-FLD were calculated against the calibration curve and subsequently corrected for the recovery (see method validation).

3.4.4 Method validation

To make comparison of the HPLC-FLD method with both LC-MS/MS methods possible, validation of both LC-MS/MS methods was performed without correction for the response of D_3BMAA.

Linearity was determined by injecting a range of calibration standards in triplicate.

For both LC-MS/MS methods, limit of detection (LOD) in calibration standards was determined as the lowest injected concentration with a signal-to-noise (S/N) ratio of all product ions of at least 3:1. Furthermore, the ratio of the qualifier ions to the quantifier ion should be within a 20% relative range of the expected value. Limit of quantification (LOQ) was defined as the lowest injected concentration with a S/N ratio of the quantifier ion of at least 10:1. Furthermore, the ratio of the qualifier ions to the quantifier should again be within the accepted range, and the S/N ratio of the qualifier ions should at least be 3:1. For HPLC-FLD analysis, LOD in calibration standards was defined as the lowest concentration of which the peak was clearly distinguishable from the background signal. LOQ was defined as the lowest concentration that was linear on the calibration curve. Detection and quantitation limits in samples were defined in the same way as for calibration standards. Limits in samples were determined by spiking a *Dolichospermum* scum sample with different BMAA concentrations prior to extraction or hydrolysis.

Intraday precision of response and retention time was determined by injecting the highest calibration standard (1000 µg/l) in six fold. For interday precision, calibration standards were injected again in six fold on a different day and the variation of all twelve injections was considered. Inter workup precision was determined by spiking an *Dolichospermum* scum sample in six fold before extraction or hydrolysis with either D_3BMAA (both LC-MS/MS methods, 200 ng for free BMAA, 400 ng for underivatised total BMAA and 2000 ng for derivatised total BMAA) or BMAA (HPLC-FLD method, 750 ng for free BMAA and 9950 ng for total BMAA). The same sample treatment was repeated in six fold on another day and response was compared. The inter workup samples were also used for calculation of recovery, which is in this study defined as the percentage of the original signal that was recovered after sample preparation and analysis.

Since no reference material is available for BMAA, accuracy was not tested. Instead, a positive control sample (cycad seed sarcotesta) was included. The mass of cycad seed sarcotesta used for extraction and hydrolysis was lower than for the cyanobacterial samples, so the signal of the cycad seed would be close to the detection limits of the methods. Furthermore, recovery of all sample treatments was determined.
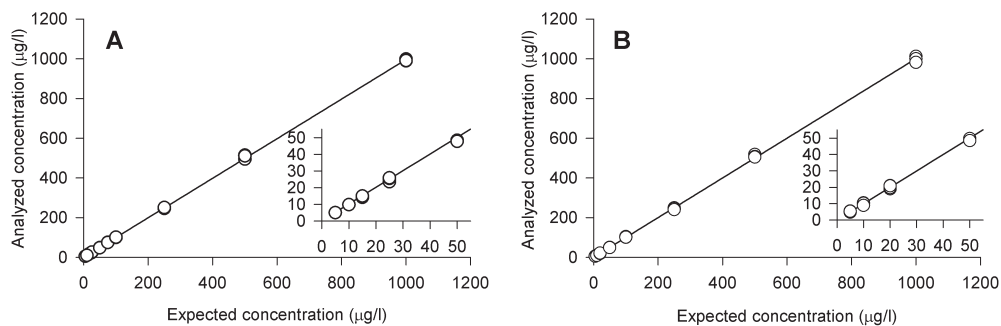
Figure A3.1. LC-MS/MS BMAA calibration curves for derivatised analysis and underivatised analysis, corrected for D₃BMAA. Panel A shows the calibration curve for derivatised analysis, panel B for underivatised analysis. All concentrations are injected in triplicate, except 5 and 10 µg/l in panel A, these concentrations are injected once.
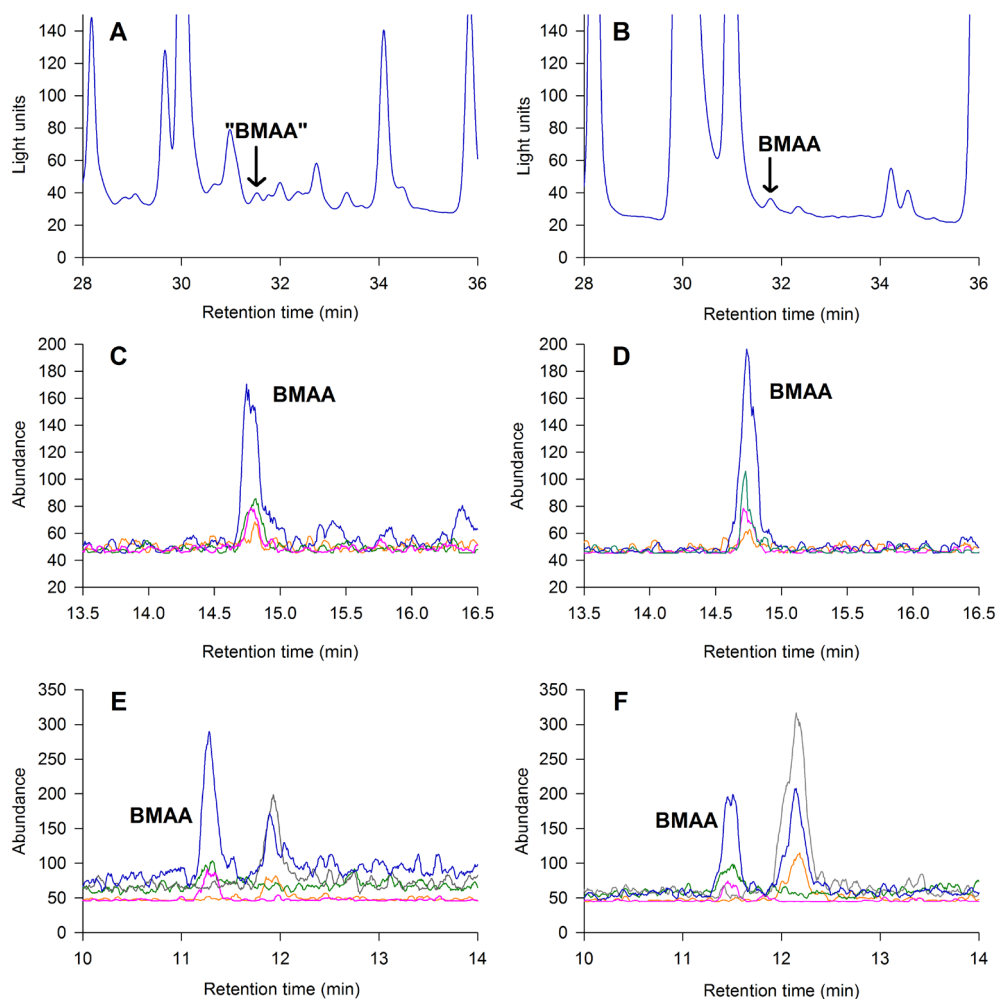
Figure A3.2. Limits of detection (LODs) for BMAA in spiked *Dolichospermum* scum samples. Panel A and B show HPLC-FLD signals, panel C and D show LC-MS/MS signals of derivatised samples and panel E and F show LC-MS/MS signals of underivatised samples. Panels C and E represent samples that are spiked with BMAA before extraction, panels B, D and F represent samples that are spiked before hydrolysis. No LOD could be defined for BMAA in extracted samples for HPLC-FLD analysis (see results in main text), panel A therefore shows an unspiked extracted field sample of *Planktothrix rubescens* with a low response at the retention time of BMAA (see also Table 3.4 in main text). Coloured lines in panels C and D represent the transitions of ions with a mass-to-charge ratio (*m/z*) of 459 to *m/z* 171 (blue), 119 (green), 145 (pink) and 315 (orange). Coloured lines in panels E and F represent the transitions of *m/z* 119.1 to *m/z* 102.1 (blue), 88 (pink), 76 (green), 101 (grey) and 74 (orange). Transitions for D_3BMAA are not shown.
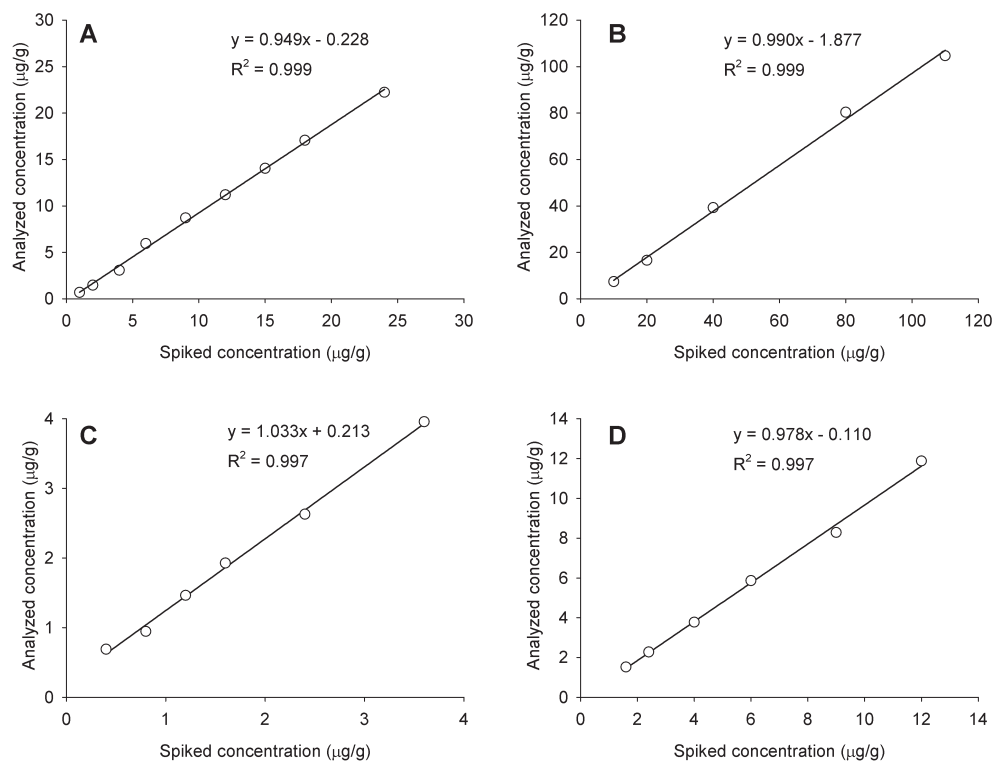
Figure A3.3. Concentrations of BMAA in spiked *Dolichospermum* scum samples, analysed by LC-MS/MS and corrected for D$_3$BMAA. Panel A shows extracted derivatised samples, panel B shows hydrolysed derivatised samples, panel C shows extracted underivatised samples and hydrolysed underivatised samples are shown in panel D. All samples are spiked before extraction or hydrolysis and are injected once.

"Blauw, blauw, blauw, blauw, blauw"

The Scene, Blauw
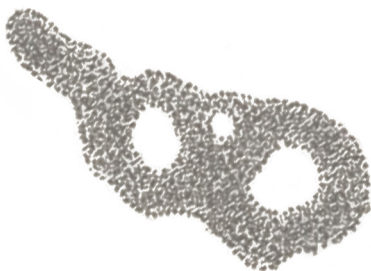
CHAPTER 4

EVALUATION OF A COMMERCIALLY AVAILABLE ELISA FOR BMAA DETERMINATION

ABSTRACT

The neurotoxin β-*N*-methylamino-L-alanine (BMAA) is suspected to play a role in Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis. Because BMAA seems to be produced by cyanobacteria, surface waters are screened for BMAA. However, reliable analysis of BMAA requires specialized and expensive equipment. In 2012, a commercial enzyme-linked immunosorbent assay (ELISA) for determination of BMAA in surface waters was released. This kit could enable fast and relatively cheap screening of surface waters for BMAA.

The objective of this study was to determine whether the BMAA ELISA kit was suitable for the determination of BMAA concentrations in surface waters. We hypothesised that the recovery of spiked samples was close to 100% and that the results of unspiked sample analysis were comparable between ELISA and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. However, we found that recovery was higher than 100% in most spiked samples; highest determined recovery was over 400%. Furthermore, the ELISA gave a positive signal for nearly each tested sample while no BMAA could be detected by LC-MS/MS. We therefore conclude that in its current state, the kit is not suitable for screening surface waters for BMAA.

4.1 INTRODUCTION

The neurotoxin β-*N*-methylamino-L-alanine (BMAA) was discovered in 1967 in cycad seeds from the island of Guam [31] and is suspected to play a role in the neurodegenerative diseases Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis [37]. Although there is proof of the neurotoxic effect of BMAA on cellular and animal level, the role of BMAA in the aetiology of these neurodegenerative diseases still needs further establishment [59]. Nevertheless, possible pathways of human exposure to BMAA are at present being investigated. After it was reported that BMAA was present in the cyanobacteria that live in symbiosis with the cycads on Guam [32], free living cyanobacteria were screened for BMAA. Initially, BMAA was detected in nearly all tested cyanobacterial species [33, 35, 36], while some later studies found lower concentrations of BMAA [83], found BMAA only in some samples [102] or did not detect BMAA at all (e.g. [76, 77]). At present, the cause of these differences in BMAA concentrations in cyanobacteria has not been identified yet, although it is very probable that studies that have used the unselective HPLC-FLD (e.g. [33, 35, 133]) have misidentified BMAA and/or overestimated its concentrations [93].

The analytical methods used for unambiguous identification of BMAA in the aquatic ecosystem should be sensitive, selective and robust [93, 134]. Methods based on tandem mass spectrometry like liquid chromatography coupled to tandem mass-spectrometry (LC-MS/MS) meet these requirements, but these types of analysis require rather specialized and expensive equipment. In 2012, a commercial enzyme-linked immunosorbent assay (ELISA) was released. The advantage of such an assay is that a rapid screening of multiple samples can be performed at relatively low costs and with relatively inexpensive equipment. The samples that give a positive signal in the ELISA then need to be further analysed by a more selective analytical method, but if the ELISA works well, time and money can be saved because the amount of samples for more specialized analysis is reduced.

The objective of our study was to determine whether the BMAA ELISA kit was suitable for the determination of BMAA concentrations in surface waters. We performed some basic tests, determined recovery of the ELISA kit in five different samples that were spiked with BMAA and we analysed unspiked water samples from different origin by ELISA and a validated LC-MS/MS method [93]. Because to our knowledge no BMAA was yet detected in untreated (i.e. not extracted or hydrolysed) water, we also included cyanobacterial extracts and hydrolysates in the experiment. We hypothesised that the recovery of the ELISA kit was close to 100% for most tested samples and that the results of the ELISA and the LC-MS/MS analysis were comparable. However, ELISA showed unexplainable deviations in the calibration curve, recoveries were higher than 100% in most spiked samples and nearly each tested sample gave a positive signal in ELISA while no BMAA could be detected by LC-MS/MS. We therefore conclude that the kit is not suitable for screening of surface waters for BMAA.

4.2 MATERIAL AND METHODS

The ELISA kits were purchased from Abraxis and are based on direct competition: BMAA competes with a BMAA-horseradish peroxidase analogue for binding sites of the rabbit anti-BMAA antibodies in solution. The BMAA antibodies are bound by a goat anti-rabbit antibody that is immobilised on the wells of the plate. The addition of a substrate generates a colour reaction that is inversely proportional to the amount of BMAA present in the sample.

In this study, we tested nine plates. First, the response of the calibration standards provided with the kit was compared to the response of calibration standards prepared in water and in sample diluent (also provided with the kit). Next, as no pH range was given in the manufacturer's instructions, we determined the response of a BMAA standard at a pH range of pH 1 to 10. We then determined recovery by spiking samples. Finally, a range of unspiked samples was analysed by ELISA and a validated LC-MS/MS method. The experiment was performed in the period from August to December 2012.

4.2.1 Calibration curves and pH series

All water used for sample preparation and analysis was purified with a Q-Pod (Millipore). BMAA calibration standards (BMAA hydrochloride, Sigma Aldrich) were prepared in water and in sample diluent (provided with the kit) directly before analysis. A pH series with 250 µg/l BMAA was constructed in a trichloroacetic acid (TCA) solution (pH range 1.4 - 3), HCl (pH range 1 - 5) and NaOH (pH range 7 - 10). The pH series was analysed in duplicate.

4.2.2 Sample collection, pre-treatment and storage

All samples except the tap water and the humic acid solutions were collected in various lakes and ponds in The Netherlands (Table 4.1). Tap water was collected in the laboratory and humic acid (Sigma Aldrich) solutions were prepared in Millipore water in the laboratory. Samples 4 and 7 were collected in a PE bottle and homogenized. A part of sample 4 was filtered over a GF/C filter (Whatman), resulting in sample 5. Sample 6 was collected by pushing a core in lake sediment. From this core, the organic top layer of the sediment was collected, centrifuged and the supernatant was filtered over a GF/C filter and collected. Samples 1-7 were stored at 4 °C. Samples 8-10 were taken from ponds and lakes with cyanobacterial blooms and were stored at -20 °C. Samples 11-14 were also taken from ponds and lakes with cyanobacterial blooms and were lyophilized before storage at -20 °C.

Dominant cyanobacterial species were identified by light microscopy. Chlorophyll-a was determined in sample 1-6 by Phyto-PAM (Walz), only sample 4 contained detectable amounts (13 µg/l) of cyanobacterial chlorophyll-a. All water samples were fresh, except for sample 7, which had an electric conductivity of 9.3 mS/cm.

No permission was required for sample collection. Samples 4, 5, 6 and 11 were collected from ponds on the campus of Wageningen University, which is private property. As employees of this university, we were allowed to enter the campus freely and to take samples for scientific research. Samples 7-10 and 12-14 were collected from lakes and ponds that were publicly accessible, which is allowed in The Netherlands. Sampling did not involve endangered or protected species and was compliant with the Dutch Flora and Fauna Act.

Table 4.1. Sample origin, pre-treatment and storage conditions.

Sample name	Origin	City	Sampling date	Cyanobacterial dominance	Pre-treatment	Storage
1 Tap water	Laboratory	n.a.	Nov 2012	n.a.	None	4 °C
2 Humic acid 10 mg/l	Laboratory	n.a.	n.a.	n.a.	None	4 °C
3 Humic acid 100 mg/l	Laboratory	n.a.	n.a.	n.a.	None	4 °C
4 No bloom unfiltered	Campus pond 1	Wageningen	Nov 2012	None	None	4 °C
5 No bloom filtered	Campus pond 1	Wageningen	Nov 2012	None	Filtration	4 °C
6 Sediment water	Campus pond 2	Wageningen	Nov 2012	None	Centrifugation and filtration	4 °C
7 Brackish	De Veste	Breskens	Nov 2012	None	None	4 °C
8 *Pl. rub.* bloom 1	Lake De Kuil	Prinsenbeek	Nov 2010	*Planktothrix rubescens*	None	-20 °C
9 *Glo. ech.* bloom	Kralingse Plas	Rotterdam	July 2012	*Gloeotrichia echinulata*	None	-20 °C
10 *Micr.* bloom 1	Urban pond	Dongen	June 2010	*Microcystis*	None	-20 °C
11 *Do.* bloom	Campus pond 3	Wageningen	June 2008	*Dolichospermum*	Lyophilisation	-20 °C
12 *Pl. rub.* bloom 2	Wuurdse Plas	Elst	April 2009	*Planktothrix rubescens*	Lyophilisation	-20 °C
13 *Aph.* bloom	Lake De Kuil	Prinsenbeek	Oct 2009	*Aphanizomenon*	Lyophilisation	-20 °C
14 *Micr.* bloom 2	Gooimeer	Almere	Sep 2009	*Microcystis*	Lyophilisation	-20 °C

n.a.: not applicable

4.2.3 Sample preparation for ELISA analysis

Directly before analysis, particles were removed from sample 8 by centrifugation and subsequent filtration over a GF/C filter. Sample 10 was also filtered over a GF/C filter and sample 9 was filtered in a tube with a 0.2 µm cellulose acetate filter (Grace Davison Discovery Science) at 16000*g.

Samples 11-14 were extracted in triplicate to release free BMAA from the cyanobacterial cells. 5 mg of sample was extracted at room temperature in the dark for two hours in 300 µl 0.1 N TCA. After the extraction, the sample was centrifuged and the supernatant was transferred. 300 µl 0.1 N TCA was then again added to the pellet and after vortexing and centrifugation the supernatant was pooled with the first supernatant. The pooled supernatant was lyophilized and then dissolved in 600 µl of water brought to pH 7 by NaOH.

The same samples were also hydrolysed in triplicate to determine total BMAA concentration. 1 mg of sample was hydrolysed in a hydrolysis/derivatisation workstation (Eldex), using 6 N HCl liquid hydrolysis for 20 hours at 105 °C in the absence of oxygen. Hydrolysates were

dried under vacuum and subsequently dissolved in 500 μl water that was brought to pH 7 with NaOH. Both fractions were diluted 5 and 10 times in water with pH 7.

pH of all prepared samples was determined with a paper indicator strip (pH-Fix 0-14, Machery-Nagel).

4.2.4. Recovery determination

Samples 1 and 4-6 were used for recovery determination. For this, they were spiked with BMAA to a concentration of 100 μg/l. Unspiked samples were also analysed and recovery (%) was determined as

$$recovery = 100 * \frac{(conc\,spiked\,sample - conc\,unspiked\,sample)}{(conc\,added\,BMAA)}$$ Equation 4.1

Extracts and hydrolysates of sample 11 were also used for recovery determination. Extracts and hydrolysates were prepared in six fold and were dissolved in sample diluent. Both fractions were diluted 5, 10, 100 and 1000 times in sample diluent. Of the undiluted extract/ hydrolysate and each dilution, three replicates were spiked to a BMAA concentration of 250 μg/l, while the other three replicates remained unspiked. Recovery was calculated for each dilution with equation 4.1. As the use of sample diluent gave problems in the recovery determination of sample 11 (see Results), recovery was also determined as described above, but the samples were dissolved in and diluted with water of pH 7.

4.2.5 ELISA procedure

ELISA kits were stored at 6 °C before analysis and were used before the expiration date. The assay was initially performed according to the instructions of the manufacturer:
- 100 μl of standard solution or sample was added to the wells
- 50 μl of enzyme conjugate solution was added with a multichannel pipette
- 50 μl of antibody solution was added with a multichannel pipette
- The plate was covered with parafilm and the plate was mixed by circular movements for 30 s on the bench top
- The plate was incubated for 90 min at room temperature in the dark
- The plate was washed four times with diluted washing solution (applied with a spray flask) and tapped dry
- 150 μl of substrate solution was added with a multichannel pipette
- The plate was covered with parafilm and the plate was mixed by circular movements for 30 s on the bench top
- The plate was incubated for 30 min at room temperature in the dark
- 100 μl stop solution was added with a multichannel pipette

- Within 15 minutes after the addition of stop solution, absorbance was read at 450 nm on a MTP reader (Synergy HT, BIOTEK).

After the first tests, we added an extra washing step with deionized water after washing with buffer solution. Furthermore, after a few tests, we replaced the sample diluent provided with the kit with water that was brought to pH 7 with NaOH for dissolving and diluting samples.

The calibration curve was constructed by fitting the equation

$$\frac{B}{B_0} = \frac{(a-d)}{\left(1+(conc/c)^{\wedge b}\right)} + d \qquad \text{Equation 4.2}$$

in Sigmaplot 12.0 (Systat Software Inc.), where B is the absorption of the calibration standard, B_0 is the average absorption of the blank (0 µg/l BMAA) and $conc$ is the concentration of the calibration standard. Parameters a, b, c and d were estimated. Samples were quantified by comparing the absorption of the sample to the absorption of the calibration curve. Samples with a signal below the signal of the lowest calibration standard (5 µg/l) were reported as not detected.

All samples except the extracts and hydrolysates were analysed in triplicate. The extracts and hydrolysates were already prepared in triplicate, so each replicate was analysed once.

4.2.6 LC-MS/MS analysis

Three series of ELISA calibration standards from different lots and samples 1-10 were prepared for underivatised LC-MS/MS analysis. Samples 1-10 were prepared in triplicate. The samples were prepared by adding 10 µl of a 10 mg/l D_3BMAA solution (internal standard) in 20 mM HCl and 640 µl acetonitrile with 0.15% formic acid to 350 µl sample.

LC-MS/MS analysis was performed according to Faassen *et al.* [93] on an Agilent 1200 LC and an Agilent 6410A QQQ. Compounds were separated on a 2.1×150 mm, 5 µm diameter ZIC-HILIC column (Sequant) with a Direct-Connect Filter (Grace Alltech). Mobile phases were acetonitrile with 0.1% formic acid (v:v, eluent A) and Millipore water with 0.1% formic acid (v:v, eluent B). Flow rate was 0.4 ml/min, injection volume 5 µl and column temperature 40 °C. The following gradient was applied: 0-2 min 5% B; 2-4 min linear increase to 35% B; 4-8 min linear increase to 45% B; 8-17 min 45% B; 17-23 min 5% B. Fragmentor voltage was 50 V and both quadrupoles were operated in unit mode. BMAA was detected by the transitions *m/z* 119.1 to *m/z* 102.1 at 4 V collision energy, *m/z* 88 and *m/z* 76 (both 8 V). Ratio of both qualifiers *m/z* 88 and *m/z* 76 to quantifier *m/z* 102.1 was 21%. D_3BMAA was detected by the transitions *m/z* 122.1 to *m/z* 105.1 (4 V), *m/z* 88 and *m/z* 76 (both 8 V). Ratio

of qualifier m/z 88 to quantifier m/z 105.1 was 22%; ratio of m/z 76 to m/z 105.1 was 37%. Calibration standards contained BMAA and D_3BMAA and were prepared in 65% acetonitrile, 35% Millipore water and 0.1% formic acid (v:v:v). BMAA concentrations in samples were determined by correcting the response of BMAA for the response of D_3BMAA.

Samples 11-14 were not analysed by LC-MS/MS in this study because they had already been analysed by LC-MS/MS previously [93].

4.3 RESULTS

4.3.1 Assay adjustment

According to the manufacturer's instructions, the plate needed to be washed with the provided washing buffer solution after the first incubation and then patted dry before the substrate solution was added. However, when this protocol was followed, lather remained in the wells, leading to large variation between replicates. We therefore added an extra washing step: after washing with buffer, the plates were washed four times with deionized water and then patted dry. When this procedure was followed, no lather remained on the plate.

4.3.2 Variation within replicates

Incidentally, a well gave a value that deviated strongly from the other two replicates without apparent reason. This happened both in the calibration curves and in the samples. Even when the person performing the test was continuously supervised by another person and no mistakes, bubbles or inaccuracies were observed while the test was carried out, these outliers kept occurring. In this study, obvious outliers were not used in the calculation of the calibration curves, but no outliers were omitted from the results.

4.3.3 Response standards

The calibration curve of the kit was S-shaped when the horizontal axis was plotted on a logarithmic scale. On three plates, the 25 µg/l standard provided with the kit showed large variation (e.g. Figures 4.1A and 4.1B). On three other plates, this standard gave an absorption close to that of the 100 µg/l standard (Figure 4.1C). The calibration standards used on one of these latter plates were analysed by LC-MS/MS and according to this analysis, the 25 µg/l standard contained the assigned concentration.

The response of the standards provided with the kit was similar to the response of calibration standards prepared in water and in sample diluent (Figures 4.1A and 4.1B). BMAA standards dissolved in acidic (TCA and HCl) and basic (NaOH) solutions ranging from pH 2.7 to 10 also gave similar results as the calibration standards provided with the kit. Below pH 2.7, their response was higher than that of the kit's standards, irrespective of whether a TCA or HCl
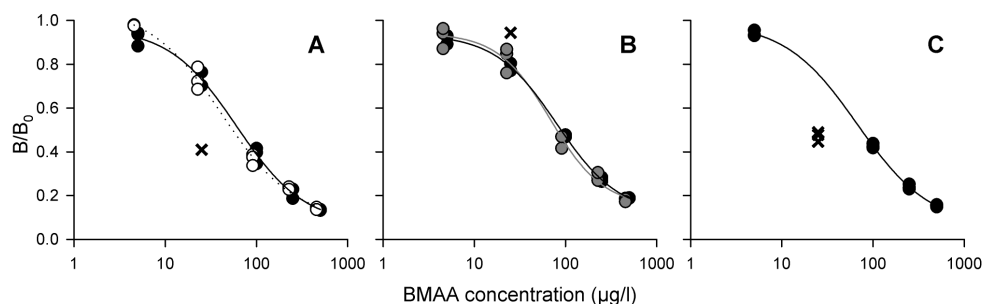
Figure 4.1. Calibration curves of three of the ELISA plates used in this study. Calibration standards provided with the kit are shown in black circles and solid black lines, calibration standards in water are shown in white circles and dotted black lines and calibration standards in sample diluent are shown in grey circles and grey solid lines. Outliers that are omitted from the calibration curve are shown as black crosses; all outliers belong to the standards from the kit.

solution was used. Therefore only samples with a pH higher than 3 were analysed in the following experiments.

4.3.4 Recovery spiked samples

Recovery was determined in four samples without cyanobacterial dominance by addition of BMAA. For all four samples, recovery was higher than 100%, recovery of the filtered sediment water was highest (408 %, Figure 4.2). pH of these samples was between 7 and 8.
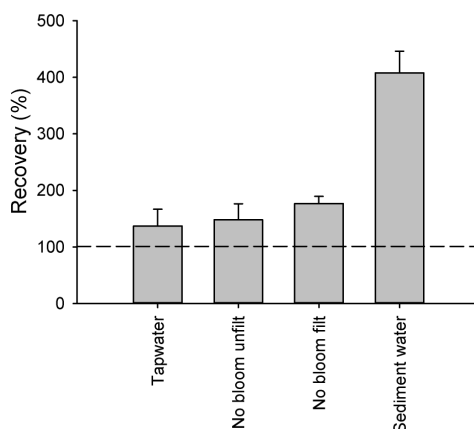
Figure 4.2. Recovery of spiked samples without cyanobacterial blooms. Error bars represent one SD, n=3.

Recovery of extracted and hydrolysed samples was determined in sample 11, a surface water with a *Dolichospermum* bloom. First, the extracts and hydrolysates were dissolved in and diluted with the sample diluent that was provided with the test. At low dilutions, recovery was higher than 100%. Only when diluted 100 and 1000 times, recovery was close

to 100% (Figure 4.3A). The pH of the undiluted extract was lower than 2 and this sample could therefore not be analysed.
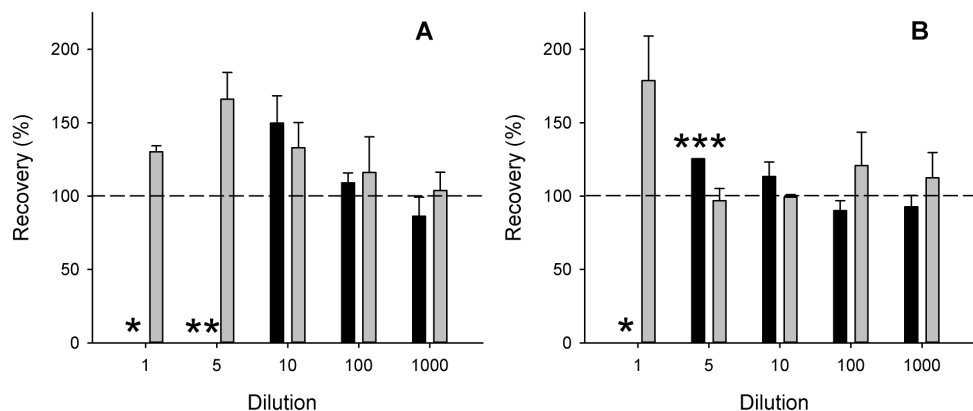
Figure 4.3. Recovery of spiked extracts (black bars) and hydrolysates (grey bars) of sample 11. In panel A, sample diluent was used as solvent and diluent, in panel B, water brought to pH 7 was used. Error bars represent one SD, n=3. *: sample not analysed due to too low pH, **: signal of all replicates above calibration curve, which corresponds to recovery >200%, ***: signal of one replicate above calibration curve, bar represents average of other two replicates.

The results of the unspiked samples that were used for the recovery determination showed inconsistencies between replicates and between different dilutions of the same replicate (Tables 4.2 and 4.3). As repetition of this part of the experiment (including renewed sample workup) did not give better results, we repeated the experiment again, but then we dissolved and diluted samples in water that was brought to pH 7 with NaOH instead of in sample diluent. Recovery of extracts that were dissolved in water with pH 7 was close to 100% when diluted at least 10 times, while hydrolysates had to be diluted at least 5 times (Figure 4.3B). The unspiked samples now gave consistent results between replicates and between dilutions (Tables 4.2 and 4.3).

Table 4.2. BMAA concentration as determined by ELISA and LOD* (both expressed as µg/g DW) for unspiked extracts of sample 11 in two different solvents.

Dilution	LOD	Solvent: sample diluent			Solvent: water pH 7		
		Replicate A	Replicate B	Replicate C	Replicate D	Replicate E	Replicate F
1	0.6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	3	14.8	a.c.	a.c.	17.6	25.6	31.0
10	6	n.d.	40.7	17.8	18.2	19.5	19.5
100	60	n.d.	n.d.	76.4	n.d.	n.d.	n.d.
1000	600	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* LOD: limit of detection, n.a.: not analysed, sample pH too low, a.c.: above calibration curve (equivalent to > 300 µg/g BMAA in sample), n.d.: not detected

Table 4.3. BMAA concentration by ELISA and LOD* (both expressed as µg/g DW) for unspiked hydrolysates of sample 11 in two different solvents.

Dilution	LOD	Solvent: sample diluent			Solvent: water pH 7		
		Replicate G	Replicate H	Replicate I	Replicate J	Replicate K	Replicate L
1	2.5	35.6	32.8	39.9	37.8	33.7	32.8
5	12.5	29.9	n.d.	78.2	35.3	40.9	39.4
10	25	n.d.	n.d.	99.4	39.7	30.5	49.3
100	250	352.4	n.d.	n.d.	n.d.	n.d.	n.d.
1000	2500	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* LOD: limit of detection, n.d.: not detected

4.3.5 Response in unspiked samples

The response of the ELISA for unspiked water samples is shown in Table 4.4 (untreated and filtered water samples) and Table 4.5 (extracted and hydrolysed water samples). Nearly all samples tested positive for BMAA, the ELISA did only not detect BMAA in some replicates of the filtered 'No bloom' sample, the sediment water sample and the 10 mg/l humic acid solution. According to the ELISA, samples 9 and 10, which are filtered samples of lakes with a cyanobacterial bloom, contained over 200 µg/l BMAA. Tap water and the humic acid solutions that were prepared in the lab also tested positive for BMAA. All cyanobacterial extracts and hydrolysates were positive for BMAA; in each sample the concentration of total BMAA was higher than that of free BMAA. No BMAA was detected by ELISA in the blanks (purified water and sample diluent).

No BMAA was detected in any of the samples by LC-MS/MS analysis in this study, neither was it detected in samples 11-14 that had been analysed by LC-MS/MS previously (field scums in Table 4 of ref [93]).

Table 4.4. BMAA concentrations (µg/l) in untreated and filtered samples as determined by ELISA.

Sample	Treatment	Average	SD	n
1 Tap water	None	12.5	6.1	3
2 Humic acid 10 mg/l	None	16.8	-	2[*]
3 Humic acid 100 mg/l	None	11.8	10.7	3
4 'No bloom'	None	10.7	2.4	3
5 'No bloom'	Filtrated	10.8	-	1[**]
6 Sediment water	Filtrated	16.5	-	1[**]
7 Brackish	None	19.9	8.4	3
8 *Pl. rub.* bloom 1	Filtrated	59.2	3.7	3
9 *Glo. ech.* bloom	Filtrated	228.9	14.8	3
10 *Micr.* bloom 1	Filtrated	298.2	42.5	3

[*]: 1 replicate not detected, [**]: 2 replicates not detected.

Table 4.5. BMAA concentrations (µg/g DW) in extracted and hydrolysed water samples with cyanobacterial blooms as determined by ELISA. Concentrations are calculated from the 10 times diluted samples.

Sample	Treatment	Average	SD	n
11 *Do.* bloom	Extraction	19.1	0.7	3
11 *Do.* bloom	Hydrolysis	39.8	9.4	3
12 *Pl. rub.* bloom 2	Extraction	30.3	4.6	3
12 *Pl. rub.* bloom 2	Hydrolysis	50.1	3.0	3
13 *Aph.* bloom	Extraction	10.5	3.7	3
13 *Aph.* bloom	Hydrolysis	85.3	11.3	3
14 *Micr.* bloom 2	Extraction	13.8	3.7	3
14 *Micr.* bloom 2	Hydrolysis	84.4	28.6	3

4.4 DISCUSSION

4.4.1 Test procedure and application range

Before starting the final experiments on samples, we adjusted the test protocol at two points: we added an extra washing step with deionized water and we used water brought to pH 7 for dilution of sample extracts and hydrolysates instead of the provided sample diluent. These adjustments made our results more reproducible and consistent. We expect that these changes in the protocol did not have a negative impact on the performance of the test. The plates were washed before the addition of colour substrate, the extra washing step with deionized water could therefore have influenced the colour reaction. However, if such an effect occurred, it has likely been equal for the calibration curves and the samples, so quantification of the samples would not be affected. We also do not expect a negative effect of the use of water for sample dilution as the kit is designed for testing water samples and because the response of calibration standards in water and sample diluents is similar (Figure 4.1). It was however surprising that the use of Millipore water brought to pH 7 gave better results than the diluent provided with the kit, as the latter consisted of distilled water according to the manufacturer.

The calibration curve of the ELISA is S-shaped, with the steepest part of the curve between 25 µg/l and 250 µg/l. Quantification in this part of the curve is most precise, below 25 µg/l and above 250 µg/l, small changes in absorbance result in relatively large variations in calculated concentrations. The manufacturer reports a level of quantification of 4 µg/l and an upper limit of 500 µg/l. Because the absorbance of the 5 µg/l standard sometimes was close to that of the blank (B/B$_0$ close to 1.0, Figure 4.1), we used a more conservative limit of detection and quantification of 5 µg/l in this study. On three plates, the 25 µg/l standard gave a signal that strongly deviated from the expected calibration curve. This was not caused by a too high BMAA concentration in these standards, as LC-MS/MS analysis confirmed that the calibration standards used on one of these three plates indeed contained the expected concentration. We therefore expect that the problem lied in the wells of the plate, or in an

impurity in the calibration standard that interfered during ELISA but not during LC-MS/MS analysis.

4.4.2 Recovery of spiked samples

The recovery of the spiked samples without cyanobacterial blooms was between 137% and 403%. As the pH of these samples was clearly above the critical threshold of 2.7, this overestimation could not be attributed to acidity of the samples. Also the possible presence of BMAA in these samples could not have caused this overestimation, as the concentrations that were determined in the unspiked samples were subtracted from the concentrations in the spiked samples. The recoveries of extracts and hydrolysates of a pond with an *Dolichospermum* bloom were also higher than 100% at the lowest dilutions. However, the recoveries of the more diluted samples were close to 100%. The mechanism behind these overestimations in spiked samples will be discussed below.

4.4.3 BMAA concentration in unspiked samples

The ELISA detected BMAA in every tested sample, although in three cases not in every replicate. No BMAA was detected by underivatised LC-MS/MS analysis in any of the samples, even though nearly all the concentrations as determined by ELISA are above the detection limit of the LC-MS/MS method [93]. LC-MS/MS analysis is considered a reliable method for BMAA detection in surface water [93, 134], although some issues have been raised against underivatised LC-MS/MS analysis [88]. However, as we think that the arguments raised by this group are refutable because we used deuterated BMAA as an internal standard [135], we consider the results of LC-MS/MS analysis reliable and therefore assume the ELISA results to be false positives.

4.4.4 Interfering compounds in ELISA

As the ELISA gave false positive results and elevated recoveries for most samples, it is likely that components in the samples have interfered. Because purified water and sample diluent contained no BMAA according to ELISA and gave accurate results when BMAA was added (Figures 4.1A and 4.1B), the problems seemed not to be caused by these solvents. One mechanism that could cause false positives and overestimation in an ELISA test is cross-reactivity: the antibody in the test does not only react with the analyte (in this case BMAA), but also with other molecules in the sample. According to the manufacturer, the BMAA ELISA shows cross-reactivity with L-cysteine hydrochloride, L-glutamic acid, L-aspartic acid (all 0.2% of BMAA signal), γ-aminobutyric acid (0.02%) and DL-2,4-diaminobutyric acid dihydrochloride (0.01%). As all of these compounds can be present in cyanobacteria (e.g. [94, 102, 136, 137]), these compounds might indeed have increased the signal. However, cross-reactivity of only these five reported compounds is unlikely to be the only cause of the frequent occurrence of false positives with sometimes high concentrations. It is likely that the test shows cross-reactivity with more compounds. From our experiments we can

identify humic acids as likely being cross reactive: a 100 mg/l humic acid solution in purified water gave a BMAA signal corresponding to 11.8 µg/l BMAA (Table 4.4).

Besides cross-reactivity, other types of interferences seemed to have occurred in our experiments. The elevated recoveries of most spiked samples cannot be explained by cross-reactivity, as the recovery calculation was based on the differences in concentration between spiked and unspiked samples. According to the manufacturer, the kit can be used in a variety of inorganic solutions and in a 10% seawater solution. The electric conductivity of the brackish sample in this study was approximately 20% of that of the neighbouring seawater, so in this sample the seawater might have interfered. However, for the other samples we do not know which mechanisms are responsible for the observed overestimation as it happened in samples that varied greatly in origin and composition. Testing for possible interferences and identifying the underlying mechanisms is a laborious task that is normally carried out during test development and we therefore considered it beyond the scope of this study.

4.5 CONCLUSIONS

The objective of this study was to determine whether the evaluated ELISA kit is suitable for determination of BMAA concentrations in surface water. To our opinion, the kit (in its current state) should not be used for this purpose. One problem with the kit is that in one third of the tested cases, no decent calibration curve could be constructed because one standard strongly deviated from the expected line. On all tested plates, outliers occurred that could not be explained by obvious errors or inaccuracies. More importantly, the test gave elevated recoveries for a diversity of spiked samples and gave false positive results in nearly all tested samples. Although the manufacturer states that the test should be used for screening purposes and that additional analytical analysis should be performed to confirm positive results, a nearly 100% score of positives in samples that are unlikely to contain detectable amounts of BMAA makes the test unsuitable for its intended purposes. As a good screening method for BMAA in surface waters can be very useful, we recommend further development of the test.

ACKNOWLEDGEMENTS

4

"I hope you're not angry if I disagree"

Eddie Vedder, Society

CHAPTER 5

PRESENCE OF BMAA IN AQUATIC SYSTEMS:
WHAT DO WE REALLY KNOW?

ABSTRACT

The neurotoxin β-*N*-methylamino-L-alanine (BMAA) is suspected to play a role in the neurological diseases amyotrophic lateral sclerosis, Alzheimer's disease and Parkinson's disease. BMAA production by cyanobacteria has been reported and contact with cyanobacteria infested waters or consumption of aquatic organisms are possible pathways to human exposure. However, there is little consensus regarding whether BMAA is present in cyanobacteria or not, and if so, at what concentrations. The aim of this review is to indicate the current state of knowledge on the presence of BMAA in aquatic ecosystems. Some studies have convincingly shown that BMAA can be present in aquatic samples at the µg/g dry weight level, which is around the detection limit of some equally credible studies in which no BMAA was detected. However, for the majority of the reviewed articles, it was unclear whether BMAA was correctly identified, either because inadequate analytical methods were used, or because poor reporting of analyses made it impossible to verify the results. Poor analysis, reporting and prolific errors have shaken the foundations of BMAA research. First steps towards estimation of human BMAA exposure are to develop and use selective, inter-laboratory validated methods and to correctly report the analytical work.

5.1 INTRODUCTION

β-*N*-methylamino-L-alanine (BMAA) is a neurotoxin that has been linked to the progressive neurological diseases amyotrophic lateral sclerosis (ALS), Alzheimer's disease and Parkinson's disease [37, 46, 59, 69]. BMAA was first discovered on the island of Guam in seeds of the cycad *Cycas micronesica* [31], which were used as food by the native Chamorro people [138]. As BMAA was shown to be neurotoxic [31], exposure to BMAA was considered as a possible cause of the high incidence of ALS/Parkinsonism-dementia complex (ALS/PDC) on this island [37]. However, the role of BMAA in the aetiology of ALS/PDC on Guam was heavily debated (e.g. [72, 139, 140]) and BMAA exposure is at present regarded as one of the possible causes of Western Pacific ALS-PDC [45].

BMAA research expanded beyond Guam after studies revealed the presence of BMAA beyond the seeds of the cycad, namely in the symbiotic cyanobacteria in the cycad's coralloid roots [32, 42] as well as in free living cyanobacteria unrelated to the cycad [33]. The possibility of a global presence of BMAA, and thus of widespread human exposure to this neurotoxin led to the hypothesis that BMAA might be related to the global presence of neurodegenerative diseases [34]. The current state of knowledge recognizes the neurotoxicity of BMAA on cellular and *in vivo* level [59, 60] but an animal model for BMAA induced ALS is still lacking [60, 66]. Recently, additional toxicity mechanisms have been proposed that might better explain the relation between BMAA exposure and the chronic nature of ALS/PDC [45, 141]. Reports of BMAA in the brain of deceased patients suffering from ALS, PDC or Alzheimer's disease support the BMAA ALS/PDC hypothesis [69-71], however, these results could not be replicated by another research group [72-74]. Some, but not all, of the differences between these studies might be tracked down to the analytical procedures applied [85].

A possible important pathway for human exposure to BMAA is through cyanobacterial blooms in water or through consumption of higher aquatic organisms exposed to such blooms [33, 35, 83]. Recently, it was reported that also planktonic diatoms and dinoflagellates contain BMAA [98, 142]. Therefore, in addition to on-going research on the role of BMAA in causing human neurodegenerative diseases, studies also focus on estimating concentrations of BMAA in aquatic ecosystems. However, reported BMAA concentrations in aquatic systems vary widely between studies. Several studies have detected BMAA in all tested cyanobacteria samples, whereas others have not detected it in any sample (Table 5.1). Furthermore, cyanobacterial BMAA concentrations vary orders of magnitude between studies (Table 5.1). Likewise, several studies have found BMAA in higher trophic levels like molluscs and fish [82, 83, 94, 97, 100, 143-146], but others have not [147, 148]. Bioaccumulation of BMAA in higher aquatic organisms has been reported [83]. However, BMAA concentrations in the two food web studies performed so far differ greatly: those reported for the Baltic sea (mostly ng/g dry weight (DW), [83]) were a few orders of magnitude lower than those for Florida (high μg/g up to mg/g DW [82]).

Table 5.1. Reported BMAA concentrations in free living cyanobacteria. Data from studies that have tested more than five independent samples are included, free and protein associated concentrations are summarized. Merged rows represent single studies. Method abbreviations are explained in Appendix A5.1.

Publication year	Quantification method	Derivatisation	n tested samples	% positive samples	[BMAA] in positive samples µg/g DW average	median	ref
2005	LC-FLD	AQC [§]	30	97	968	265	[33]
2008	LC-FLD	AQC	12	100	103	76	[35]
2008	LC-FLD	AQC	7	100	10	7.3	[107]
2008	GC-MS	EZ:faast	27	96	130	3.5	[36]
2008	LC-MS/MS *	none	34	0	-	-	[76]
2009	LC-MS/MS	none	21	43	13	6.0	[102]
2010	LC-MS/MS ^	none	30	0	-	-	[77]
2010	LC-MS/MS	AQC	21	100	0.01	0.01	[83]
2011	LC-MS	EZ:faast	20	80	1.4	0.49	[122]
2011	CE-UV	none	8	100	402	277	[121]
2012	LC-FLD	AQC	18	100	14	9.0	[133]
2012	LC-FLD	AQC	16	100	0.29	0.24	[133]
2012	LC-MS/MS #	AQC	8	0	-	-	[93]
2012	LC-MS/MS ~	none	8	0	-	-	[93]
2012	LC-FLD	AQC	8	38	28	22	[93]
2014	LC-MS/MS	AQC	10	100	4.4	3.2	[149]

* Limit of detection (LOD) free < 1 µg/g DW, LOD total < 4 µg/g DW, ^ LOD 1.0 µg/g DW, # LOD free 1 µg/g DW, LOD total 10 µg/g DW, ~ LOD free 0.4 µg/g DW, LOD total 1.6 µg/g DW. [§] AQC: 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate

A possible explanation for the striking variations in BMAA concentrations (Table 5.1) could be that BMAA is produced in detectable amounts in some cyanobacteria and not in others. Concentrations of cyanobacterial secondary metabolites can vary within species, between species and between locations (e.g. [29, 128, 150]) but the variation within studies is usually larger than the variation between studies (e.g. [151, 152]) – though this is not the case for the BMAA results reported. Indeed, there is a strong bimodality in the absence/presence of BMAA in cyanobacteria samples, and analysis of similar [93, 133] or comparable samples [36, 122] with different methods yields different results (Table 5.1). This strongly suggests that additional factors to those influencing cyanobacterial metabolite production play a role in the reported divergences in cyanobacterial BMAA concentrations. In fact, the use of non-selective analytical methods likely is a major cause of the observed differences between studies [93], as is discussed in the next section. Additionally, even in cases where the appropriate analytical techniques are used, many research articles contain reporting errors such as an incomplete description of methods and results. In this setting, it is difficult to tell when BMAA has in fact been detected, as is shown in section 5.4. Furthermore, the absence of critical discussions in many studies hinders the comparison of data and findings, as is shown in section 5.5.

The objective of this review is to elucidate the current state of knowledge on the presence of BMAA in aquatic ecosystems, based on studies in which appropriate analytical techniques have been employed and that were correctly reported. For this, I analysed primary research articles on the analysis, occurrence and production of BMAA in phytoplankton and higher aquatic organisms. Moreover, in the Appendices, I discuss some key articles on BMAA analysis, BMAA production by cyanobacteria and human exposure through cyanobacteria to illustrate the effect of reporting errors in their context (Appendices A5.2 to A5.6).

The main outcome of this review is that there is evidence for the presence of BMAA in aquatic organisms, but that this evidence is only based on a fraction of the published work. The assumed widespread occurrence of BMAA in aquatic ecosystems and its production by cyanobacteria could, therefore, not be verified. I find that unclear reporting and unsupported conclusions in key articles have shaken the foundations of BMAA research, an issue that needs to be tackled to determine human BMAA exposure routes and to provide a solid fundament for follow up studies.

5.2 THE ROLE OF ANALYTICAL METHODS IN THE BMAA CONTROVERSY

The use of different analytical methods in BMAA research has recently extensively been discussed [93, 134], and is summarized in this section, since it plays an important role in explaining observed differences in BMAA concentrations.
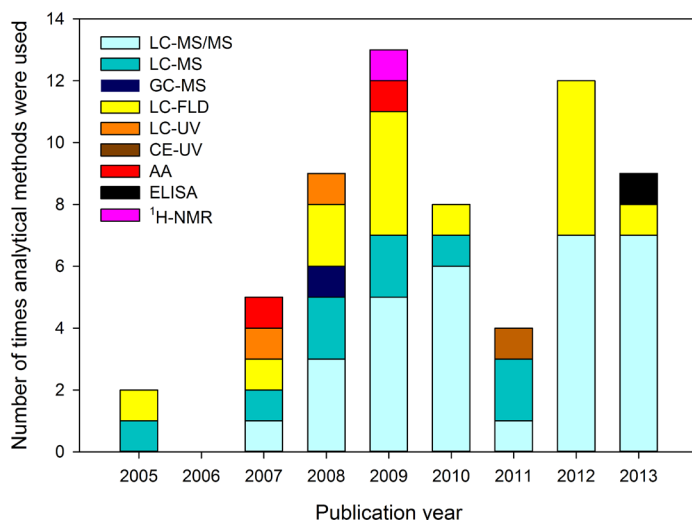

Figure 5.1. Analytical methods used for determination of BMAA concentrations in the aquatic ecosystem from 2005 up to 2013. Method abbreviations are explained in Appendix A5.1.

The most selective analytical methods used for BMAA analysis are ^{1}H-NMR and LC-MS/MS. ^{1}H-NMR was only used in one study [111], but the sensitivity of this method is very low (LOD

5 mg/l). LC-MS/MS is the most frequently applied technique (Figure 5.1), and it is selective because it relies on four criteria for the identification of analytes (retention time, mass-to-charge ratio (*m/z*) of the parent ion, *m/z* of product ions after collision induced dissociation and ratio between these product ions). Therefore, the chance of misidentification is minimized.

In LC-MS and GC-MS, no collision induced dissociation is used and these methods are, therefore, less selective than LC-MS/MS. In LC-FLD, LC- or CE-UV and AA analysis, analytes are identified by retention time and optical signal. As only two identification criteria are used by these methods, they are regarded as non-selective. An analytical technique that is based on different principles than the ones discussed before is ELISA, in which antibodies are used for identification. Recently, an ELISA for BMAA determination in environmental samples became commercially available. However, this test was shown to be unsuitable for its intended use [153].

A recent review on analytical techniques for BMAA research [134] recommends the use of MS/MS instead of optical methods (e.g. FLD) for detection. Indeed, there is a substantial chance of misidentification and overestimation of BMAA concentrations with optical detection based methods, as has experimentally been shown for one LC-FLD method [93]. In that study, FLD analyses resulted in overestimation or false positives in three out of eight tested samples. A group of scientist argued that BMAA could be successfully separated from its isomer α-,γ-diaminobutyric acid (DAB) [84] and other diamino acids [95] by a diversity of analytical methods, including those with optical detection. However, they do not properly answer the most important questions of possible misidentification by optical methods. It is unclear whether BMAA was separated from DAB in six of the ten investigated methods, because the chromatograms showing separation, or their legends are incomplete or incorrect (Appendix A5.2). Furthermore, only a narrow selection of possibly interfering compounds was tested, while in real samples many more compounds could possibly interfere (Appendix A5.3). Finally, it is not clear whether BMAA was correctly separated from the tested compounds in earlier studies by these authors, as it is not explicitly stated how the presented results relate to previous work (Appendices A5.2 and A5.3).

MS/MS is currently generally accepted as the preferred detection technique for BMAA analysis, but when combined with LC separation, this technique also has its drawbacks. A main concern with LC-MS and LC-MS/MS analyses is the possible loss of signal by ion suppression [85, 88], when sample components other than the analyte decrease (and in some cases also enhance) the analyte signal [154]. The severity of this effect should, therefore, be estimated and reported for each LC-MS(/MS) method [85].

To enhance its compatibility with different analytical methods, BMAA is sometimes derivatised. Derivatisation is used to change the properties of BMAA, e.g. to enhance its volatility for GC-MS analysis, to add chromophores for optical detection or to reduce polarity for reversed phase LC separation. While derivatisation adapts analytes to each technique, it does not necessarily influence the selectivity of each method. Therefore, the observed differences in cyanobacterial BMAA concentrations are not related to whether or not samples were derivatised (Table 5.1). The observed differences cannot either be explained by lack of sensitivity, as the detection limits of most methods with which no BMAA was detected were generally below the average concentrations found by others (Table 5.1).

Optical detection methods are still used in BMAA research (Figure 5.1). However, in the context of European guidelines for pesticide residue analysis, these techniques are only acceptable for frequently found residues – and always in conjunction with additional confirmatory methods – but more selective methods like MS/MS are preferred [155]. Identification by single MS is only regarded reliable when two or more diagnostic ions are used [155].

5.3 REVIEW OF REPORTED METHODS AND RESULTS

As detailed above, BMAA can only be reliably detected if the appropriate methods are used. Furthermore, for results to be clear and comparable, it is essential to report methods and results adequately. I here evaluate the methods and results sections of studies on BMAA detection in aquatic ecosystems. For each method, I checked if the following basic information was well reported: sample origin and storage, sample processing, sample analysis, method performance and BMAA identification.
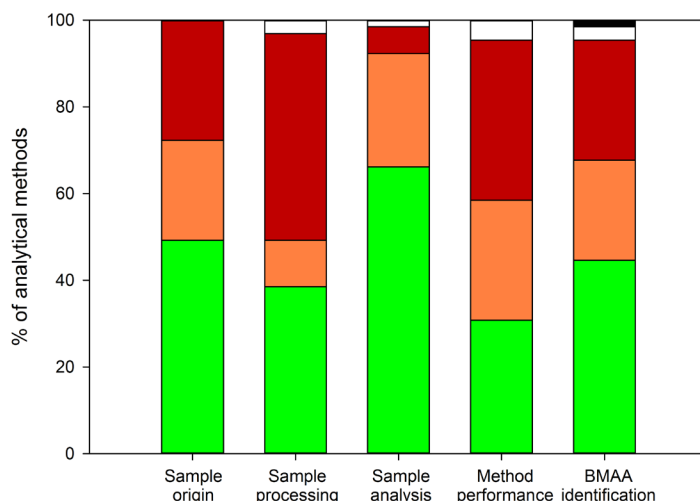

Figure 5.2. Quality of reporting for analytical methods. Bars indicate: (nearly) correct and complete (green), incomplete or with errors (orange) and absent or with major errors (red). Data that have been published in previous method descriptions are shown in white, and 'not applicable' is shown in black. Results are summarized from Appendix A5.7.

5.3.1 Sample origin and storage

Sample origin and storage conditions were well described in nearly half of the performed analyses (Figure 5.2). Most of the studies for which no data on sample origin and storage were provided focused on method development, but five studies focused on BMAA detection in cyanobacteria [33, 156-159]. Especially for these latter studies, information on sample origin and storage is required for the right interpretation of the detected BMAA concentrations, as cyanobacterial amino acid and toxin concentrations can change with changing growth conditions (e.g. [128, 150, 160, 161]).

Incomplete description of sample origin in combination with incomplete data presentation can undermine the conclusions of a study. For instance, in a study on exposure of Gulf War veterans to BMAA [156], samples were taken from different locations in the Qatar desert. It is unclear how many samples were taken at each location, and which of these samples contained BMAA. The amount of BMAA in each sample was also not reported. As it unclear which locations of the Qatar desert contain which amount of BMAA, it is difficult to estimate the possible exposure of the veterans. The suggestion that BMAA exposure through desert dust may be linked to the increased incidence of ALS in Gulf War veterans is, therefore, not supported by the presented data (Appendix A5.4).

5.3.2 Sample processing

Essential information on sample processing was lacking for half of the analyses (Figure 5.2), and it was impossible to estimate the workup efficiency for most of these analyses. Most details were lacking on volumes and weights during sample processing and on the derivatisation protocol (Appendix A5.7). Information on volumes and weights is required because volume-to-weight ratios partly determine extraction efficiencies, and the amount of sample injected in LC-MS(/MS) can influence the signal strength during analysis. Furthermore, derivatisation efficiency is also dependent on the sample/reagent ratio [125, 134]. The derivatisation procedure was only sufficiently described sixteen times, while derivatisation was used in 49 analyses (Appendix A5.7). For some analyses, an estimation of the total sample processing efficiency (including the derivatisation step and/or clean-up) could be derived from the use of internal standards and/or recovery data (e.g. [36, 122, 135, 143, 162]). For most analyses, however, it remains unclear whether derivatisation was efficient and, therefore, whether BMAA concentrations were correctly determined or underestimated.

5.3.3 Sample analysis

In most studies the sample analysis was well described (Figure 5.2), but information on quantification was often missing (Appendix A5.7). For 18 out of the 43 analyses in which BMAA concentrations were determined, it was unclear how this was done. Quantification can be performed in different ways, e.g. against a calibration curve of pure standards or

spiked matrices, and with or without correction for internal standards or recoveries. Different methods of quantification can give different results, so this information is essential to allow comparison of studies.

When analytical methods are poorly described, they cannot be reproduced by other scientists and the results of the study cannot be validated by independent replication. For instance, some methods could not be reproduced because it was unclear how the elution programs was performed [96, 98] and for another study, it was even impossible to tell which analytical procedure had been followed [146].

5.3.4 Method performance

Method performance and validation data are used to show that the applied method is suitable for its intended purpose [130]. However, for most analyses these data were incomplete or missing (Figure 5.2). For instance, recovery was only correctly reported for one third of the methods (Appendix A5.7). Unless internal standards are used, recovery data should be used to correct the analysed BMAA concentration for possible losses during processing and/or analysis. One of the studies for which no recovery data are available is the study on BMAA concentrations in the Baltic food web [83]. The cyanobacterial BMAA concentrations found in this study are by far lower than those found in other studies (Table 5.1). The validity of these results cannot be evaluated, because recovery data are neither given in the article in which the study is described [83], nor in the methodological article that preceded this study [94].

For sixteen methods, most data needed for method validation (detection limits, linear range, precision and recovery) were provided [34, 76, 79, 82, 93, 94, 100, 105, 111, 121, 122, 135, 145, 148]. However, unvalidated methods (or methods for which no sufficient validation data were provided) were repeatedly referred to as 'validated'. This was mainly the case for one LC-FLD method [34, 82, 84, 95, 107, 144, 158]. In addition, it was also stated that 'the' AQC based method for BMAA analysis has been validated by other methods [88]. However, it is unclear what 'the' AQC based method is, as AQC derivatisation has been used in combination with many different analytical methods (e.g. Table 5.1). In the only studies where multiple AQC methods were quantitatively compared, there was a discrepancy in results between the AQC LC-FLD and the AQC LC-MS/MS method [93] and between two AQC LC-FLD methods [133].

5.3.5 BMAA identification

Correct identification of BMAA can be shown by comparing a sample chromatogram (for methods using optical and mass spectrometry detection) or spectrum (mass spectrometry) to that of a BMAA standard or a sample spiked with BMAA. In 27 out of 65 methods, chromatograms or spectra provided enough information to prove that BMAA was correctly

identified (Appendix A5.7). For 21 methods, BMAA identification could not be verified because no (six methods) or only one chromatogram/spectrum was shown (fifteen methods, Appendix A5.7) and the response of a sample could not be compared to that of a standard. For other methods, chromatograms were incorrectly displayed.

For LC-MS/MS analysis, the four analyte identification criteria (retention time, *m/z* of the parent ion, *m/z* of product ions after collision induced dissociation and ratio between these product ions) should be shown to be the same between a BMAA standard and BMAA detected in a sample. However, for many LC-MS/MS analyses this was not correctly demonstrated: none of the studies that used LC-MS/MS to confirm positive findings by LC-FLD [34, 35, 82, 107, 144, 156, 158, 159, 163] reported the LC-MS/MS identifications correctly. LC-MS/MS identification was only correctly presented in studies that used LC-MS/MS as their primary method [76, 77, 79, 93, 94, 97, 98, 100, 102, 135, 145, 164]. Examples of incorrectly displayed LC-MS/MS identifications are spectra of standards and samples acquired at different collision energies [82, 156], which makes them incomparable. Furthermore, the ratio of the product ions between the BMAA standard and the sample differed in two studies [142, 144], in other studies the integration method used for the different product ions was inconsistent [143, 163] and different BMAA retention times between spiked pure water and a spiked cyanobacterial extract were shown without explanation [105].

There are also problems with the proof of correct identification of BMAA in the only GC-MS study on cyanobacterial BMAA concentrations [36]. Chromatograms of standards, spiked and unspiked samples were provided, but the peaks in the unspiked samples that were attributed to BMAA did not exceed the noise level (Figures 5.3B and 5.3C). Furthermore, the spectrum of the standard differed from the spectra of the samples (Figure 5.3), so additional fragments could not be used to confirm presence of BMAA, as is required in single MS analysis [155].

5.4 BIAS THROUGH SELECTIVE LITERATURE REFERENCES AND LACK OF DISCUSSION

As described in the previous sections, it is for many studies unclear whether BMAA is correctly detected and quantified. In addition, literature interpretation is hindered by a lack of critical reflection on the quality and limitations of some studies. Also, certain studies selectively cited only literature on positive findings of BMAA, thereby creating a biased view on the subject.

5.4.1 Selective use of references

The selective use of references has in some studies resulted in a biased view towards positive findings of BMAA. For the first articles on BMAA in cyanobacteria [33, 35, 36], knowledge on presence of BMAA in cyanobacteria was limited and the results of these studies were in
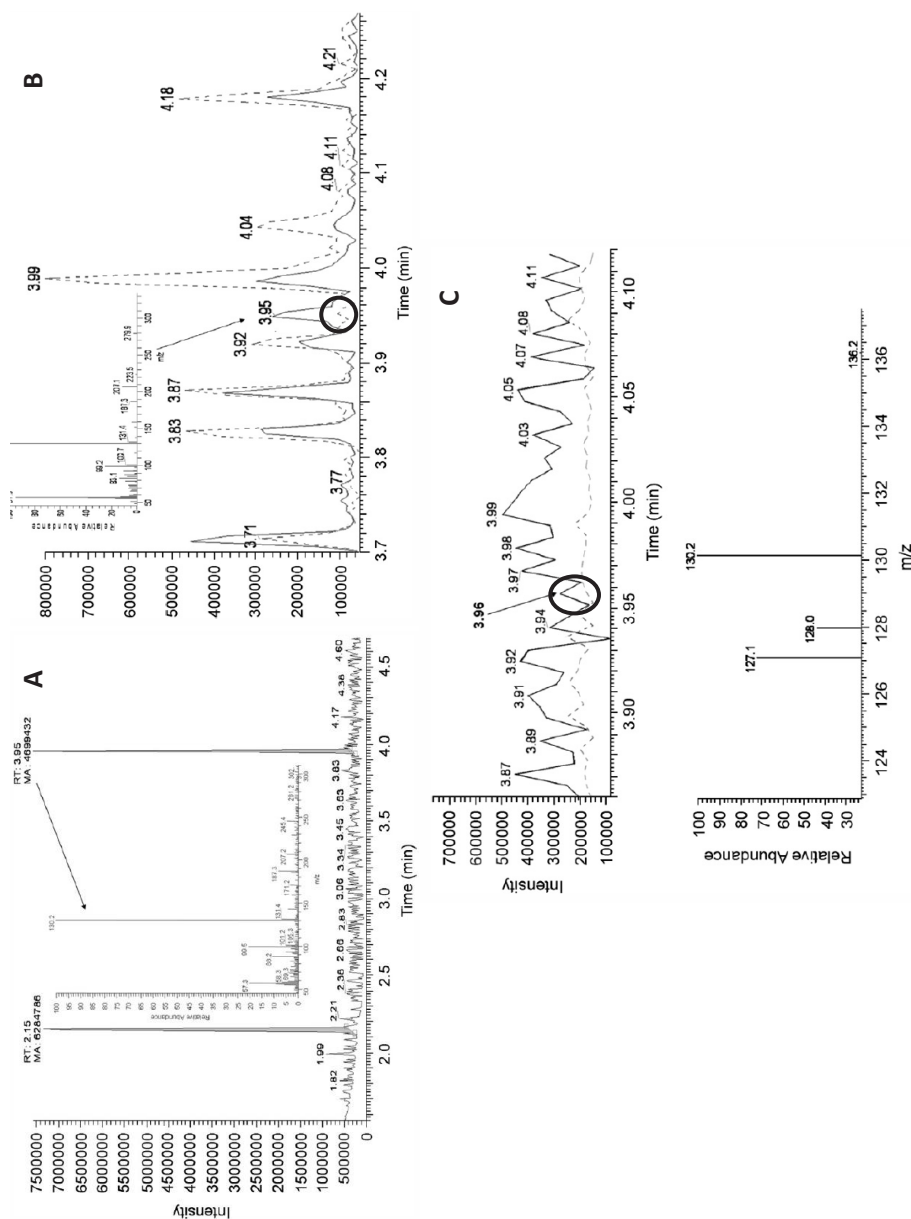
Figure 5.3. GC-MS chromatograms and spectra of standards, spiked and unspiked cyanobacterial samples as published in [36]. Circles indicate detection of unspiked BMAA and are added in this review. **A** Chromatogram of standard BMAA (Retention time (*Rt*) 3.95 min; *m/z* 130.2) measured against an internal standard of norvaline (*Rt* 2.15 min, *m/z* 158, 72), **B** GC-MS detection of BMAA (dotted line) detected from strain R202.1 spiked with an authenticated BMAA standard (Sigma, solid line) **C** Comparison of BMAA detection using a lyophilized (dotted line) culture and fresh (solid line) culture (strain Q411.1 *Leptolyngbya*). Reprinted from [36], with permission from Elsevier.

agreement with each other. However, results started to differ when no BMAA was detected in subsequent studies [76, 78, 111]. As a consequence, a public discussion on the suitability of the applied analytical methods and the correctness of results started (e.g. [76, 77]). These conflicting data and/or methodological issues were mentioned in most articles from 2009 and later, but were ignored by some (e.g. [82, 95, 144, 147, 157, 159, 163]). Especially in studies in which optical detection was used as the primary analytical method and in which high BMAA concentrations were found using these methods [96, 121, 133, 144]; no reference to the debate on concentrations and methods was made.

Also in an experimental study on method performance [88], a biased view was created by selective referring to previous research. In this study, adduct formation in underivatised LC-MS analysis was investigated and it was concluded that for this type of analysis, adduct and complex formation may lead to an extreme underestimation of BMAA concentrations. However, the authors do not adequately discuss the underivatised LC-MS or LC-MS/MS publications in which complex and adduct formation do not seem to play a major role. Neither do they refer to the only study in which underivatised and derivatised LC-MS/MS analyses were directly compared, and in which underivatised LC-MS/MS performance was slightly better than derivatised LC-MS/MS performance. Finally, the authors recommend using derivatised LC-MS analysis with LC-FLD as a confirmatory technique, but do not discuss the points raised against the use of LC-FLD for BMAA analysis in several other publications (Appendix A5.5).

5.4.2 Discussion of quality and limitations of the study

In most articles, presented work was not critically discussed and limitations of the study were rarely addressed, which contributes to uncertainty about the validity of some results. For instance, one group published an article on derivatisation optimization [125] which was followed by a methodological article on SPE and LC-MS/MS analysis of samples [94]. This method was subsequently used for a food web study [83]. In 2012, the same group published two more methodological articles, one on separation of BMAA from isomers [97] and one describing quantification by LC-MS/MS [135]. These articles have greatly contributed to aquatic BMAA research, but on the same time give rise to some questions. For instance, why was an optimum ratio between sample protein and derivatisation reagent advised in the first study [125], and this ratio by far exceeded in the second study [94]? Furthermore, the LC-MS/MS method was adjusted in 2012 because the ratio between product ions used for BMAA identification in samples did not always correspond to the ratio in a BMAA standard [97]. If these ratios do not correspond it is uncertain whether BMAA is present in the samples. It was concluded that the difference in ratios might have been caused by an interfering isomer, but the question regarding whether this interference was also present during the food web study [83] and, therefore, whether BMAA was correctly identified in this study was not addressed. Finally, a subsequent article by this group described an

optimized LC-MS/MS method that could be used for quantification [135]. Sensitivity was improved in comparison to the first published method when expressed as fmole/injection (70 in [94] and 4.2 in [135]), but it is not discussed why, when expressed in µg/g dry weight, this method was a hundred times less sensitive (LOD of 0.1 µg/g DW) than the first method [94] by which a concentration of 0.001 µg/g DW had been detected [83].

Similarly, another group published several articles on method development and sample testing [36, 122, 162] and one on BMAA production by cyanobacteria [99]. In two of these articles, BMAA concentrations were determined in multiple cyanobacterial isolates. Although the tested isolates were not identical, they were described in both articles as being representative for the region and they were cultured under similar conditions [36, 122]. The average BMAA concentration determined by GC-MS in one study [36] was nearly a hundred times higher than the average concentration determined by LC-MS in the other study [122] (Table 5.1), but possible causes of this difference were not adequately discussed [122]. Furthermore, from the experimental study [99], the authors conclude that cyanobacteria produce BMAA in response to nitrogen starvation. The authors suggest that some other studies did not detect BMAA because only nutritionally replete cyanobacteria were analysed. However, the authors do not check this with their previous work, in which they frequently reported BMAA in cyanobacteria that were grown on BG11 [36, 122], a medium that is very rich in nitrogen [165, 166].

5.5 CONCLUSIONS

5.5.1 Presence of BMAA in aquatic ecosystems

There is evidence that BMAA can be present in cyanobacteria dominated samples [83, 94, 102, 135, 164], while in some equally credible studies, BMAA has not been detected in cyanobacteria [76-79, 93, 97]. The evidence for presence of BMAA is generated by studies that have used LC-MS/MS, which is at present regarded as one of the most suitable techniques for BMAA analysis due to its high selectivity and sensitivity [93, 134]. In addition, these studies have correctly shown the appropriate chromatograms, so it is likely that BMAA has been correctly identified. The negative results are based on well reported LC-MS and LC-MS/MS studies. LC-MS is less selective than LC-MS/MS, but this method is included because less selective methods do not have a higher risk of creating false negative results than more selective methods.

Based on studies in which the quantification method has also properly been reported, BMAA concentrations in positive cyanobacterial samples are 0.73 µg/g DW in a cyanobacteria culture [135] or range from 4 to 42 µg/g DW in field material dominated by, but not necessarily solely comprised of, cyanobacteria [102]. These concentrations are close to the detection limits of the previously mentioned LC-MS(/MS) studies in which no BMAA was detected, which range from 0.1 to 10 µg/g DW [76-78, 93, 97] (LOD in [79] is not included

because it is expressed per unit of wet weight). No BMAA was detected in cyanobacterial samples by ^{1}H-NMR, another highly selective, but rather insensitive method (LOD of 5 mg/l) [111].

One LC-MS/MS based study has convincingly shown that axenic diatom cultures can contain BMAA at concentrations between 1.1 and 3.3 ng/g DW, the BMAA concentration in a cyanobacteria/diatom dominated field sample was 27.6 ng/g DW [98].

BMAA has also been detected in (some samples of) higher aquatic organisms [83, 94, 97, 100, 145], at concentrations between 4.7 and 14.1 μg/g DW in crabs [145], 6.8 and 46.9 μg/g DW in oysters [145] and 0.63 and 1.6 μg/g wet weight in mussels [100] by LC-MS/MS based studies. The two studies in which no BMAA was detected in higher aquatic organisms (fish and shrimp) have used LC-FLD analysis (LOD 0.21 μg/g DW for fish and 0.3 μg/g DW for shrimp [147, 148]).
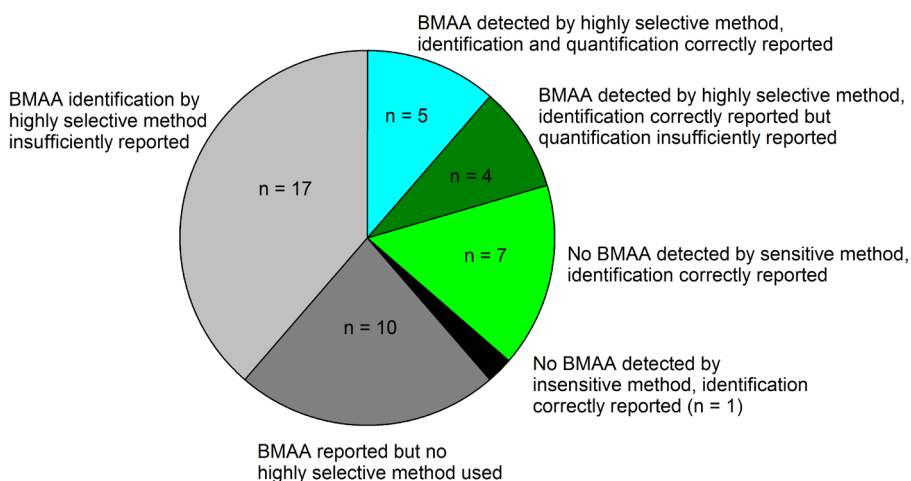
Figure 5.4. Number of studies that have provided convincing evidence for correct BMAA analysis in aquatic samples (blue and green slices) and studies that have not provided conclusive evidence (grey and black slices). Classification is explained in Appendix A5.8.

Although it is shown that BMAA can be present in aquatic ecosystems, this conclusion is only based on a narrow selection of articles (Figure 5.4). There is too little evidence to conclude that BMAA is occurring worldwide in aquatic ecosystems. Independent confirmation from a number of different laboratories is needed to verify this hypothesis.

More work is also needed to identify the BMAA producers in aquatic systems. Only one study has confirmed presence of BMAA in an axenic cyanobacterial culture [94]. The only study so far on BMAA production by cyanobacteria [99] does not provide evidence as the

experimental setup is flawed, there is too little evidence for correct BMAA identification and not all results are presented (Appendix A5.6). Diatoms might also be BMAA producers, as one study has shown that axenic diatom cultures contain BMAA [98]. Taken together, only two studies have identified cyanobacteria and diatoms as possible BMAA producers, but their findings have not yet been confirmed by other, independent laboratories. The question whether other organisms than phytoplankton are capable of BMAA production has so far only been explored for cycads [80].

For the majority of the published work on BMAA in aquatic ecosystems, it is unclear whether the results were correct. The field of BMAA analysis is still developing, and lack of certified reference material and an inter-laboratory validated method has resulted in the use of different analytical techniques. Amongst these, non-selective analytical methods have frequently been used, which may have caused false positives or overestimations. In addition, the appropriateness of some selective analytical methods could not be verified because BMAA identification was insufficiently reported.

Nevertheless, the use and description of analytical techniques are not the only sources of confusion in aquatic BMAA research. It lacks a solid foundation as the conclusions drawn in some of the key articles on analysis [84, 88, 95], production by cyanobacteria [99] and human exposure through cyanobacteria [156] were either unclear or not supported by the presented data. Finally, literature interpretation is hindered by occasional selective referencing to positive findings and by lack of a critical evaluation of the presented work.

5.5.2 Improving the science

The first steps to advance BMAA research in aquatic ecosystems have been made by the increased use and development of selective analytical methods. An inter-laboratory comparison and validation of these methods, preferably using certified reference material, would be a next step toward a more unified discussion on which analytical methods to use in BMAA research. However, the field can only move forward if the use of appropriate methods is combined with correct and complete description of research. Future studies should include an extensive and correct description of methods and results (see e.g. [85]), with special attention to recovery data, quantification procedure and identification. Furthermore, the work should be critically evaluated and should be put it in its scientific context.

A solid base of knowledge relies on good insight into past results. Most evidence for correct BMAA analysis is provided in sixteen studies [76-79, 83, 93, 94, 97, 98, 100, 102, 135, 145, 147, 148, 164]. While it is well possible that BMAA has correctly been identified (and/or quantified) in other studies, there is no publicly available evidence for it. Full analysis

disclosure, or availability of sample material for comparative analyses is essential to improve the current state of knowledge in BMAA research.

In conclusion, the current knowledge on presence of BMAA in aquatic ecosystems is more limited than the literature suggests. The state of knowledge will increase if appropriate, inter-laboratory validated methods are developed and used, and if the analytical work is correctly reported. This progress is needed to establish to what extent humans are exposed to BMAA through for instance ingestion of surface water during recreation and consumption of seafood.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

I am the first author of three of the articles evaluated in this review [93, 102, 153]. I declare no other conflict of interest.

5

AA (amino acid analyser):
liquid chromatography separation, detection by visible light absorption

CE-UV:
capillary electrophoresis separation, ultra violet detection

ELISA (enzyme linked immunosorbent assay):
identification by antibodies, detection by visible light absorption

GC-MS:
gas chromatography separation, mass spectrometry detection

^{1}H-NMR:
proton nuclear magnetic resonance spectroscopy

LC-FLD:
Liquid chromatography separation, fluorescence detection

LC-MS:
Liquid chromatography separation, mass spectrometry detection

LC-MS/MS:
Liquid chromatography separation, tandem mass spectrometry detection

LC-UV:
Liquid chromatography separation, ultra violet detection

APPENDIX A5.2.

ARTICLE DISCUSSION 'DISTINGUISHING THE CYANOBACTERIAL NEUROTOXIN β-*N*-METHYLAMINO-L-ALANINE (BMAA) FROM ITS STRUCTURAL ISOMER 2,4-DIAMINOBUTYRIC ACID (2,4-DAB) [84]'

From 2008 up to 2010, it was repeatedly suggested that DAB had by some methods possibly been misidentified as BMAA (e.g.[76, 77]). In this 2010 article, LC and GC based methods are reviewed and their ability to separate BMAA from DAB is discussed.

LC separation of BMAA and DAB

The LC section starts with an overview of methods that can distinguish DAB from BMAA, and chromatograms are shown to prove separation. However, the legends of six of the ten provided chromatograms are incomplete or incorrect, so it is unclear by which methods the chromatograms are produced:

- Fig 2A: Legend refers to a 60 min gradient in [43], but in this reference a 49 minute gradient was employed
- Fig 2C: Legend refers to studies in which no BMAA analysis was performed [167, 168]
- Fig 4: Sample used from [156], method unknown. Retention time of BMAA does not match the retention time in [156]. Figure shows BMAA and DAB, while in [156] only BMAA was shown in the LC-MS/MS chromatogram
- Fig 6: No method given, this seems to be a later published method [122]
- Fig 7: No method given
- Fig 8: DAB not shown, no methods given but figure is identical to Fig 1 in a later published study [162]. Legend states that samples have been derivatised according to [36], but [36] is not a LC-MS but a GC-MS study in which a different derivatisation procedure has been used. This is reflected by the different reported *m/z* for the BMAA derivatised: 130.2 in [36] and 333 in this figure.

The authors suggest that negative findings by underivatised Hydrophilic Liquid Interaction Chromatography (HILIC) MS/MS analysis are caused by inferior performance of this method. However, they did not compare method performance data like LODs and recoveries to verify whether the performance of underivatised methods was indeed worse than that of derivatised MS/MS methods. An example is given to support the claim that HILIC separation is less efficient and results in broad peaks, however the peak that is referred to (Figure 2A in [77]) is of equal width as one of the peaks shown for derivatised separation (Figure 2A in [84]). Furthermore, authors state that DAB was routinely used in sample queues for LC methods from the first article in 2003 on [42], but in many of the articles published after 2003 by the authors, DAB is not mentioned (e.g. [33, 35, 107]). Furthermore, they state

that negative findings of another study [78] were confirmed by the author's own LC-MS/MS methods, but no method details or references are provided.

GC separation of BMAA and DAB

The question whether BMAA and DAB are separated in GC based methods [36, 169] is not answered in this manuscript, although there is a paragraph dedicated to this subject. It is only stated (in the LC paragraph) that in one GC-MS method [36], BMAA is separated from alanine, sarcosine and trypotophan. However, the retention times of these three amino acids have not been reported in [36]. Instead, they are taken from another study in which different conditions are used [170]. As different chromatographic conditions can lead to differences in retention times, the results of [170] cannot be extrapolated to [36]. Furthermore, it was stated that a GC-MS method developed by another group [169] might be too insensitive for BMAA detection, however only the LOD for the protein-bound fraction is referred to, and not the LOD for free BMAA, which was 50 times lower [169].

Discussion

In the discussion, the authors recommend a comparative exercise between laboratories that should include a repetition of validated methods (for issues with the term validated, see Section 5.3.4 in this Chapter), a balanced view on whether HILIC chromatography is adequate for BMAA analysis and a check for unambiguous determination of BMAA. They hereby again question the suitability of HILIC based methods without providing fact based arguments against it.

In conclusion

This article suffers from many reporting deficiencies: not all addressed questions were answered, parts were suggestive and incorrect references were given. Methods that had not yet been published were included in this review, although it was stated that previous results were considered. Furthermore, separation of DAB and BMAA by GC-MS was not adequately discussed. As it is not made explicit which studies have and which have not separated BMAA from DAB, the conclusion that these compounds are "routinely and clearly distinguished by having different retention times during chromatographic separation" is not supported by the presented data.

In this 2011 article, a group of scientists reports the separation of BMAA from other diamino acids. Five analytical techniques are described and chromatograms showing separation of BMAA from other compounds are presented.

Justification and research aim

It is unclear why this research has been performed, it lacks a description of the justification and aim. In the introduction, authors only state that it is important to distinguish BMAA from similar molecules. The method section does not provide clarity either. It consists of the description of five analytical techniques, all but one are not referred to, and are therefore assumed to be developed for this study. The one method referred to (LC-FLD, § 2.7.), should also be regarded as a newly developed method. Although the references [34, 42, 43] suggest it has already been used before, the elution program used in two of these studies differs from the one described in § 2.7. [34, 43]. In the other study [42] no elution program was reported, but a reference to yet another study [126] is made. The elution program in this reference does however also not correspond to the elution program described in § 2.7.

Relevance of the work

After having reached this point in the manuscript, it looks like new methods were developed to enable good separation of BMAA from other diamino acids. When placed in the context of the main debates in BMAA research from 2009 to 2011, some questions on the relevance of the work arise:

- The interference in methods based on optical detection is not limited to diamino acids, but to compounds with amino groups [76, 93]. Why are only a few diamino acids tested for these methods?
- Diamino acids with a different molecular weight from BMAA are not the most likely candidates to interfere in methods with mass-spectrometry detection [93]. Why are all but two tested diamino acids compounds with a different molecular weight?

Relation to previous and future work

In the results section, authors state that the methods have indeed been adapted in this study and show that BMAA can be distinguished from the tested diamino acids by these methods. It then, however, becomes confusing whether the subsequent conclusions are based on methods used in this study or on previously used methods. Authors state that standard LC-MS/MS methods distinguish BMAA from other amino acids, with a reference to previously

used methods [34, 94]. As these previous methods differ from the ones presented in this study, this statement would imply that the conclusion is based on previous methods, but this conclusion is not supported by data or chromatograms. Furthermore, it is mentioned that the BMAA isomer *N*-2(amino)ethylglycine coeluted with BMAA in the current LC-UV/MS method, but that in previous studies that included LC-UV or LC-MS analysis [34, 92, 107, 156], BMAA identification was correct because other methods were used for confirmation. Also in this case, three [34, 92, 107] of the four the methods referred to are different from the tested method so it is unclear whether *N*-2(amino)ethylglycine coelution had also occurred in these studies. In the discussion "standard methods" are mentioned without definition: "We found that standard methods of amino acid analysis clearly distinguish BMAA from the twelve amino acids tested".

The repeated referring to "standard" and "previously used" methods implies that BMAA was already separated from other diamino acids in previous work. As it is neither discussed how the methods used in this study relate to previously used methods and nor whether, to the opinion of the authors, BMAA was correctly identified in previous studies, the implications of the study remain unclear.

In conclusion

It is unclear which questions are answered by this study. Five methods have been shown to separate BMAA from a selection of diamino acids, but the relevance of this selection is questionable and not discussed. As these methods have all been adapted in this study, no conclusion about separation of BMAA from the selected compounds in previous studies can be drawn. However, the repeated reference to "standard" and previously used methods could mistakenly make a reader think that it is shown in this article that BMAA was also separated from the selected compounds in previous work.

Appendix A5.4.
Article discussion 'Cyanobacteria and BMAA
exposure from desert dust: A possible link to
sporadic ALS among Gulf War veterans [156]'

In this 2009 article, cyanobacteria crusts from the Qatar desert were collected and analysed for BMAA in order to assess whether veterans had been exposed to BMAA during the Gulf War. It was concluded that BMAA was present in the cyanobacteria crusts, which led to the suggestion that BMAA exposure through desert dust may be linked to the increased incidence of ALS in Gulf War veterans.

Incomplete description of methods

Cyanobacteria crust samples were taken from one location in 2007 and from three locations in 2008. It is unknown how many samples were taken from each location. After sampling, some crust samples were dried and analysed, while others were cultured and subsequently analysed. It is unclear how many samples were cultured and what the origin of this/these samples was.

Four different methods were used for BMAA analysis. It is unclear which samples were analysed by which methods. The only thing that is clear is that samples that were positive for BMAA in AA and/or LC-FLD analysis were reanalysed by LC-MS/MS and that the cultured samples were analysed by LC-MS and AA.

Incomplete description of results

Authors state in the Results section that BMAA was detected in desert crust samples from one location and that BMAA and DAB were detected in the cultured crust. It is unknown whether BMAA was detected in the samples from the other locations. The provided chromatograms do not provide more clarity as is not made clear to which samples they belong, it is only stated whether they represent dried or cultured samples. Furthermore, some samples were analysed by multiple methods, but the results of these analyses per sample are not presented, so it is unclear whether they are in agreement. BMAA concentrations are not reported.

Incorrect data visualisation

The provided LC-MS/MS chromatogram consists of three panels, one of which gives information on the sample and the other two give information on a BMAA standard. In two of these panels, product ions for a standard and for a sample are shown. However, the product ions of the standard were acquired at different settings than those of the sample. This results in different ratios of product ions between the standard and the sample (see

also Section 5.3.5 of this Chapter). It is unclear whether these ratios would have been the same when analysed under the same conditions and therefore whether BMAA has correctly been identified by LC-MS/MS.

In conclusion

The major problem of this article is that it is unclear how many samples were analysed and how much BMAA was present in each sample. As the presence of BMAA in cyanobacteria was linked to BMAA exposure of veterans, it is essential to know the BMAA concentrations and distribution in the sampled areas. Another problem is the confirmation by LC-MS/MS. The crust samples that tested positive for BMAA with the less selective methods LC-FLD and AA were reanalysed by the more selective method LC-MS/MS. However, the presented LC-MS/MS chromatograms do not provide enough information to prove that BMAA was correctly identified with this method.

5

This 2012 article explores a possible explanation for the frequent lack of detection of BMAA by underivatised LC-MS(/MS) analyses. For this, the effect of different solutions on the BMAA signal in LC-MS analysis is determined. The authors hypothesise that the formation of adducts and complexes hinders electrospray ionisation MS analysis and can distort chromatography.

Adduct and complex formation and the detection of mass-to charge ratio (*m/z*) 119

Adduct formation is determined by adding BMAA to different trichloroacetic acid (TCA)/salt solutions. These mixtures are scanned at different cone voltages and the intensity of the ion with a *m/z* of 119 (singly charged BMAA) and the *m/z*'s of BMAA adducts and dimers are recorded. As the response of *m/z* 119 is low in most solutions and for most settings, it is concluded that even under optimal circumstances, *m/z* 119 accounts for less than 10% of the total BMAA ions in solution. This is however contradicted by Figure 2A, which shows a 70% presence of *m/z* 119 and by Figure 1, which shows a 30% presence. Furthermore, this conclusion implies that other MS settings are optimized for BMAA analysis at *m/z* 119 and that TCA is the optimal solution for BMAA detection. This is contradictory to earlier work, which is not discussed in the current article. In this earlier work, *m/z* 119 is found to be the main peak after infusion of an aqueous BMAA solution [76].

Complementary to the evaluation of adduct formation, the authors explore whether metal complexes of BMAA can form in sample matrixes by chemically synthesizing a BMAA-Zinc complex. However, chemical synthesis of a BMAA-Zinc complex does not prove that this complex will be formed in real samples. To prove this, real samples should be analysed for the presence of such a complex, but this is not done.

Implications for sample analysis

The authors conclude that signal suppression and alteration of chromatographic behaviour due to adduct and complex formation may lead to an extreme underestimation of BMAA concentrations in underivatised LC-MS sample analysis, especially when external calibration curves are used for quantification. However, nearly all underivatised MS(/MS) studies have anticipated this by determining recovery [77, 78, 93, 102, 120], by using matrix based calibration curves [79] or by using D₃BMAA as an internal standard [76, 93]. In these studies, recovery rates generally exceed 80% and matrix based calibration curves have similar slopes as neat curves, so there is no indication that strong signal suppression indeed occurs

in real samples. The authors, however, suggest that the reaction time used for recovery determination is too short for complex formation, and that reported recoveries are therefore highly overestimated. This is an important suggestion, as it supports the main conclusion of the article. However, it is not grounded on arguments or data. In the method section, it is not mentioned that BMAA was allowed to react with TCA or salt solutions for a given period of time, nor are data on reaction time shown in the results section. Also in an older article that describes BMAA-metal complex formation [87], no reaction times are given.

Furthermore, the authors suggest that the solutions used in this study are representative for sample extracts, but they do not provide data on metal concentrations in cyanobacterial extracts. Trace element composition of marine phytoplankton [171], however, suggest that metal concentrations in cyanobacterial extracts will be much lower than the concentrated (9 mM salts and 10 mM TCA for each treatment) solutions used in this study. The authors conclude that BMAA reactivity may complicate analysis of many different types of samples, but have not verified this conclusion by analysing real samples.

Recommended analytical procedure

The article ends with the recommendation to use derivatisation combined with reversed phase chromatography for sample analysis, and to use at least two orthogonal detection methods such as FLD and MS. Finally, it is recommended to use multiple *m/z*s in BMAA analysis. This latter recommendation is supported by the presented work, but the other recommendations are not. Firstly, no adequate comparison between derivatised and underivatised MS analysis is carried out in this study. In the only study in which these methods have been compared, derivatised MS/MS analysis did not perform better than underivatised MS/MS analysis [93], but the authors do not refer to this study in their discussion. Furthermore, FLD detection is suggested as additional technique, without referring to recent articles that provide arguments against the use of this method [93, 134].

In conclusion

A possible weak point in MS analysis is pointed out in this article, which is valuable. However, the relevance of the experiments for real sample analysis is not shown and most conclusions are not supported by data. Finally, not all relevant publications are discussed, resulting in a biased view on the subject.

Appendix A5.6.
Article discussion 'Nitrogen
starvation results in the production of
β-*N*-methylamino-L-alanine [99]'

This 2011 article was the first to investigate conditions under which cyanobacteria produce BMAA. In this experimental study, two cyanobacterial strains are repeatedly subjected to nitrogen starvation. The presence of BMAA in the cultures is monitored throughout the experiments and authors conclude that nitrogen starvation results in the production of BMAA.

Flaws in experimental setup

One weak point of this work is that the design of this experiment is flawed. Nitrogen is supplied as labelled ammonium in the experimental treatment and as unlabelled nitrate in the control treatment. As cyanobacteria differ in their response to ammonium and nitrate as nitrogen source [172], the type of nitrogen source should be similar between treatments. Furthermore, in a nitrogen starvation experiment, the proper control treatment would be nitrogen repletion rather than nitrogen starvation.

Suboptimal analysis

Another problem with this study is the detection of BMAA. Samples were analysed by LC-MS/MS, either as a Q1 scan without collision induced dissociation or in multiple reaction monitoring mode with collision induced dissociation and detection of product ions. This means that the LC-MS/MS was used as a less selective LC-MS for most of the analyses.

Presentation of raw data

The presented data are either too little or too much processed for good interpretation. Examples of too little data processing are the figures in which a BMAA decrease or increase is shown. In these figures (Figure 1 and 3), results are presented as LC-MS(/MS) peak areas instead of as cellular or biomass related toxin content or concentrations, which are more commonly used (e.g. [150, 173]). During the experiment, a fixed volume of sample was taken at each sampling event. It is not shown how the biomass concentration changed during the experiments, but as the experiment is performed in batch cultures, it is likely to have changed. Therefore, it is reasonable to assume that different amounts of cyanobacteria were present in the different samples. As only the intensity of the MS(/MS) signal is shown, no correction is made for these changes in biomass concentration. Figures 1 and 3, therefore, only reflect the total amount of BMAA present in the sample and it cannot be derived whether the observed changes in these figures reflect changes in cellular

BMAA concentrations or merely result from changes in biomass concentration during the experiment.

Obscured data presentation

The changes in labelled amino acid abundance in the experimental cultures are too much processed to allow easy interpretation. The increases in labelled amino acids after nitrogen starvation are shown as ratios of the singly labelled to the unlabelled amino acids, which are subsequently normalized against control cultures. This use of ratios of ratios obscures the results. More importantly however, results do not represent a response to nitrogen starvation as the control treatment had also been nitrogen starved. The results represent the differences in cyanobacterial response to the nitrogen sources used and from the presented data it can again not be derived whether these differences are caused by changes in cellular composition and/or in biomass.

Incomplete data presentation

Not all essential data are presented. Data on biomass indicators and nutritional status of the cyanobacteria are lacking. The table that shows the increase in labelled amino acids, lacks data for some samples, but this is not explained. In addition, Figures 1 and 3 only show free unlabelled BMAA, the unlabelled protein associated fractions and both fractions of labelled BMAA are missing.

In conclusion

This study suffers from flaws in the experimental design and lack of data on cyanobacterial biomass, nutritional status and presence or absence of different BMAA fractions. It was for instance not checked whether the cyanobacteria were really nitrogen starved and the right control treatments were not included. Furthermore, the employed LC-MS/MS was mostly operated as a LC-MS without motivation. Too little data (e.g. chromatograms with product ions of standards and a samples) were provided to show that BMAA was correctly identified. Taken together the above mentioned flaws and omissions and either the lack of data processing or the expression of data as ratios of ratios instead, this article's conclusions cannot be verified by the presented data.

Table A5.7. Reporting quality of methods and results. Only methods with which environmental samples were tested are included. Symbols used are: + (nearly) correct and complete, ~ incomplete or with errors, - absent or with major errors, p shown in previous publication of the same method, x not applicable. Coloured columns summarize previous columns, colours correspond to the symbols + (green), ~ (orange), – (red) and x (white). Studies in which previously published method were used and referred to are indicated by: § [94], ^ [148], # [93] and % [98].

ref	method	Pre column derivatisation	Sample origin	Growth conditions/moment of sampling	Storage conditions	Sample origin and conditions	Volumes and weights	Derivatisation protocol	Processing repeatable	Hardware described	Method described	Method of quantification	Analysis repeatable	Cal curve/linearity	LOD/LOQ* defined	LOD/LOQ standard reported	LOD/LOQ sample reported	Precision	Recovery	Method performance	Chrom/spectrum standard	Chrom/spectrum sample	Chrom/spectrum spiked sample	BMAA identification
[33]	LC-MS	Y	~	-	-	-	-	-	-	+	-	x		-	-	-	-	-	-		-	-	-	
[33]	LC-FLD	Y	~	-	-	-	-	-	-	~	~	~	~	-	-	+	-	-	~	~	+	+	-	+
[35]	LC-FLD	Y	+	+	+	+	-	-	-	+	+	-	~	-	-	+	-	-	-	-	+	+	+	+
[35]	LC-MS/MS	Y	+	+	+	+	-	-	-	+	+	x	~	-	-	-	-	-	-	-	-	~	-	-
[34]	AA	N	+	-	-	~	+	x	+	+	+	-	+	-	-	-	-	-	-	-	-	+	-	~
[34]	LC-MS/MS	Y	+	-	-	~	-	-	-	+	~	~	~	-	-	-	-	-	-	-	-	+	-	~
[34]	LC-MS	Y	+	-	-	~	~	~	~	+	+	x	+	-	-	-	-	-	-	-	-	~	~	-
[34]	LC-UV	Y	+	-	-	~	+	+	+	~	+	~	+	+	-	+	-	+	-	~	-	~	~	~
[34]	LC-FLD	Y	+	-	-	~	+	+	+	+	+	+	+	-	-	-	-	-	-	-	~	~	~	~
[125]	LC-FLD	Y	+	+	+	+	~	-	-	+	~	+	~	~	+	~	~	-	-	~	-	-	~	-
[78]	LC-MS	N	~	-	-	-	+	x	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+
[107]	LC-UV	Y	+	+	-	~	~	-	-	~	~	-	~	-	-	-	-	-	-	-	+	+	-	+
[107]	LC-MS	Y	+	+	-	~	~	-	-	+	+	x	+	-	-	-	-	-	-	-	+	+	-	+
[107]	LC-MS/MS	Y	+	+	-	~	-	-	-	+	+	x	+	-	-	-	-	-	-	-	-	~	-	-
[107]	LC-FLD	Y	+	+	-	~	~	-	-	+	+	~	+	-	-	+	-	-	-	~	+	+	-	+
[36]	GC-MS	Y	+	+	+	+	-	-	-	+	+	+	+	+	-	+	-	-	-	~	+	~	~	~
[76]	LC-MS/MS	N	~	-	-	-	~	x	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+
[111]	^{1}H-NMR	X	~	-	-	-	~	x	~	~	+	+	+	+	+	+	-	+	+	+	+	-	+	+
[148]	LC-FLD	Y	~	-	-	-	+	+	+	+	+	x	+	+	+	+	+	-	+	+	+	+	+	+
[102]	LC-MS/MS	N	+	+	+	+	+	x	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+
[157]	LC-MS	Y	+	-	-	-	-	-	-	~	-	~	-	-	-	+	-	-	~	~	-	-	-	-
[158]	LC-MS/MS	N	~	-	-	-	-	x	-	+	~	-	~	-	-	-	-	-	-	-	~	~	~	~
[158]	LC-FLD	Y	~	-	-	-	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-
[163]	LC-MS/MS	Y	+	+	+	+	+	-	~	+	+	x	+	-	-	-	-	-	-	-	-	-	-	-
[159]	LC-MS/MS	Y	-	-	-	-	+	-	-	+	+	x	+	-	-	-	-	-	-	-	-	+	-	~
[156]	AA	N	-	+	-	-	-	x	-	+	+	x	+	-	-	-	-	-	-	-	-	+	-	~
[156]	LC-MS/MS	Y	-	+	+	-	-	-	-	+	+	x	+	-	-	-	-	-	-	-	~	~	-	-
[156]	LC-FLD	Y	-	+	+	-	-	-	-	+	~	x	~	-	-	-	-	-	-	-	+	+	-	+

Table A5.7. Continued

ref	method	Pre column derivatisation	Sample origin	Growth conditions/moment of sampling	Storage conditions	Sample origin and conditions	Volumes and weights	Derivatisation protocol	Processing repeatable	Hardware described	Method described	Method of quantification	Analysis repeatable	Cal curve/linearity	LOD/LOQ* defined	LOD/LOQ standard reported	LOD/LOQ sample reported	Precision	Recovery	Method performance	Chrom/spectrum standard	Chrom/spectrum sample	Chrom/spectrum spiked sample	BMAA identification
[156]	LC-MS	Y	-	+	-	-	-		-	+	~	x	~	-	-	-	-	-	-	~	+	+	-	+
[120]	LC-MS/MS	N	+	+	-	~	~	x	-	+	+	x	+	+	+	+	-	+	~	+	~	~	-	-
[120]	LC-MS	N	+	+	-	~	~	x	-	+	+	x	+	+	+	+	-	+	~	+	+	+	-	+
[120]	LC-MS/MS	Y	+	+	-	~	~	-	-	+	+	X	+	-	-	-	-	-	-	-	-	-	-	-
[94]	LC-MS/MS	Y	+	+	+	+	~	+	+	+	+	+	+	-	+	+	-	+	-	~	+	+	-	+
[83]	LC-MS/MS§	Y	+	+	+	+	~	+	+	+	+	+	+	-	p	p	-	p	-	p	+	+	-	+
[82]	LC-FLD	Y	+	+	~	+	+	-	~	~	+	+	+	+	-	-	+	-	+	~	-	+	+	+
[82]	LC-MS/MS	Y	+	+	~	+	-	-	-	+	~	x	~	-	-	-	-	-	-	-	~	~	-	-
[77]	LC-MS/MS	N	+	+	+	+	+	x	+	+	+	+	+	-	+	-	+	-	+	~	+	+	+	+
[122]	LC-MS	Y	+	+	+	+	-	-	-	+	+	~	+	+	+	+	-	+	-	+	+	+	-	~
[99]	LC-MS/MS	Y	+	+	+	+	-	-	-	+	+	x	+	-	-	-	-	-	-	-	-	+	-	~
[133]	LC-FLD	Y	+	+	~	+	~	+	+	+	~	-	~	-	+	+	-	+	-	~	~	-	-	-
[121]	CE-UV	X	+	+	~	+	~	x	+	+	+	+	+	+	+	-	+	+	+	+	+	-	~	~
[162]	LC-MS	Y	-	-	+	-	~	-	-	+	+	~	+	-	-	-	-	-	+	~	-	~	-	-
[147]	LC-FLD^	Y	+	+	+	+	+	+	+	+	+	x	+	-	-	+	-	-	+	+	p	p	p	p
[79]	LC-MS/MS	N	+	+	+	+	+	x	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+
[96]	LC-FLD	Y	+	-	-	-	+	+	+	+	-	-	-	+	+	+	-	~	+	+	+	+	+	+
[144]	LC-FLD	Y	+	~	+	+	-	-	-	~	+	~	~	-	-	+	-	-	~	~	+	+	+	+
[144]	LC-MS/MS	Y	+	~	+	+	-	-	-	-	+	x	~	-	-	-	-	-	-	-	~	~	-	-
[97]	LC-MS/MS	Y	~	-	+	-	~	-	-	+	+	x	+	-	+	-	+	-	+	~	+	+	+	+
[93]	LC-MS/MS	N	+	+	+	+	+	x	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
[93]	LC-MS/MS	Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
[93]	LC-FLD	Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
[145]	LC-MS/MS	Y	+	-	-	-	~	+	~	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
[143]	LC-MS/MS	Y	~	+	+	~	-	-	-	~	~	-	~	+	+	+	+	+	+	+	~	~	+	~
[135]	LC-MS/MS	Y	+	+	-	+	~	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	~
[105]	LC-MS/MS	N	+	+	+	+	+	x	+	+	+	+	+	+	+	+	+	+	+	+	+	-	~	~
[153]	ELISA	X	+	+	+	+	+	x	+	+	+	+	+	+	+	+	+	+	+	+	x	x	x	x
[153]	LC-MS/MS#	N	+	+	+	+	+	x	+	+	+	+	+	p	p	p	p	p	p	p	p	p	p	p
[146]	LC-MS/MS	Y	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
[100]	LC-MS/MS	Y	+	-	+	~	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
[164]	LC-MS/MS§	Y	+	+	-	~	p	p	p	+	~	x	+	-	-	-	-	-	-	-	+	+	-	+
[149]	LC-MS/MS	Y	+	+	~	+	~	-	~	+	~	-	~	-	-	-	-	-	+	~	-	-	-	-
[174]	LC-FLD	Y	+	~	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	~	-	-	+	~
[174]	LC-MS/MS	Y	+	~	+	+	+	+	+	+	+	-	~	+	-	+	-	-	+	~	-	-	+	~
[98]	LC-MS/MS	Y	+	+	+	+	~	-	~	+	~	+	~	+	-	-	-	-	-	-	+	+	-	+
[142]	LC-MS/MS%	Y	~	p	p	+	p	p	p	p	p	p	p	p	p	p	p	p	p	p	+	~	-	~

* Limit of detection (LOD)/Limit of quantification (LOQ)

Table A5.8. Criteria used for the classification in Figure 5.4 of the main text.

Group*	Positive results for BMAA reported	At least one highly selective method used	Sensitive method used	BMAA identification correctly reported	Quantification correctly reported	References
1	+	+	+	+	+	[98, 100, 102, 135, 145]
2	+	+	+	+	-	[83, 94, 97, 164]
3	-	n.a.	+	+	n.a.	[76-79, 93, 147, 148]
4	-	+	-	+	n.a.	[111]
5	+	-	n.a.	n.a.	n.a.	[33, 36, 96, 121, 122, 125, 133, 153, 157, 162]
6	n.a.	+	n.a.	-	n.a.	[34, 35, 82, 99, 105, 107, 120, 142-144, 146, 149, 156, 158, 159, 163, 174]

*Group numbers indicate:
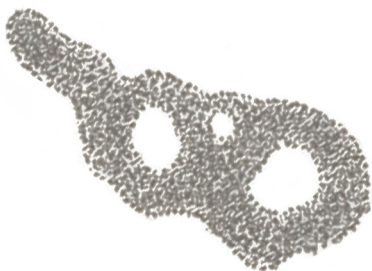
1: BMAA detected by highly selective method, identification and quantification correctly reported

2: BMAA detected by highly selective method, identification correctly reported, but quantification insufficiently reported

3: No BMAA detected by sensitive method, identification correctly reported

4: No BMAA detected by insensitive method, identification correctly reported

5: BMAA reported but no highly selective method used

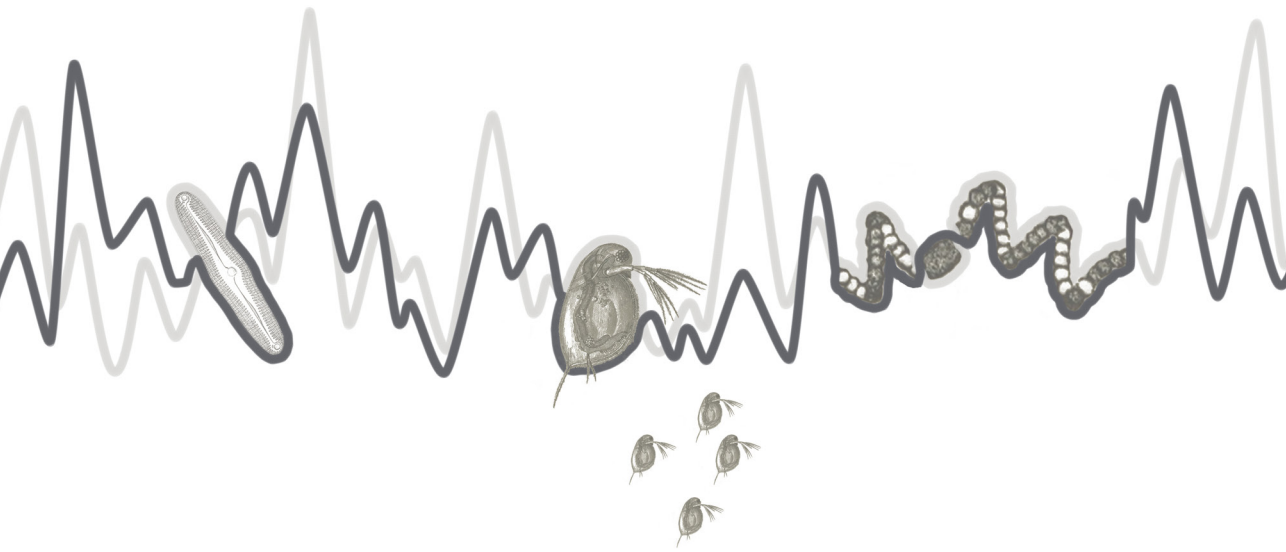6: BMAA identification by highly selective insufficiently reported

n.a.: not applicable.

"…"

Kyteman, Sorry

CHAPTER 6

Trans generational effects of BMAA on *Daphnia magna*

ABSTRACT

β-*N*-Methylamino-L-alanine (BMAA) is a neurotoxin that is suspected to play a role in the neurological diseases amyotrophic lateral sclerosis, Alzheimer's disease and Parkinson's disease. BMAA has been detected in phytoplankton and globally, the main exposure routes for humans to BMAA are through direct contact with phytoplankton infested waters and consumption of BMAA contaminated fish and invertebrates. As BMAA can be transferred from mothers to offspring in mammals, BMAA exposure is expected to have trans generational effects. The aim of our study was to determine whether maternal exposure to BMAA affects offspring fitness in zooplankton. We performed a multi-generational life history experiment and a multi-generational uptake experiment with the water flea *Daphnia magna* as a model species. In both experiments, offspring from non-exposed and exposed mothers were raised in clean and BMAA containing medium. Direct exposure to 110 µg/l BMAA reduced survival, somatic growth, reproduction and population growth. Maternal exposure did not affect *D. magna* fitness: animals from exposed mothers that were raised in clean medium had a higher mortality and produced lighter neonates than the controls, but this did not result in lower population growth rates. No evidence for adaptation was found, instead, multi-generation exposure to BMAA had a negative effect: animals that were exposed during two generations had a lower brood viability and neonate weight than animals born from unexposed mothers, but raised in BMAA containing medium. Maternal transfer of BMAA was observed, but BMAA concentrations in neonates raised in BMAA containing medium were similar for animals born from exposed and unexposed mothers. Our results indicate that zooplankton might be an important vector for the transfer of BMAA along the pelagic food chain, but whether BMAA plays a role in preventing zooplankton from controlling cyanobacterial blooms needs further investigation.

6.1 INTRODUCTION

The neurotoxin β-*N*-methylamino-L-alanine (BMAA) is suspected to play a role in the neurological diseases amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease [37, 46, 59, 69]. BMAA was discovered on the island of Guam in seeds of the cycad *Cycas micronesica* [31], which were used as food by the native Chamorro people [138]. Since there was a long latency period between exposure and onset of the disease, BMAA was considered to be a slow toxin [175]. It was suggested that this latency period was caused by the presence of a 'toxic reservoir', in which BMAA was stored in a protein-associated form and was slowly released in its free form [70]. Recent studies indeed indicate that BMAA can either associate with, or be incorporated into proteins [65, 90, 141, 176], although this association might in some cases be superficial [91]. BMAA can be maternally transferred as shown for mice, either directly [177] or trough milk during lactation [178]. If BMAA can reside inside the body for a longer period of time and can be transferred to offspring, it is likely that BMAA exposure can have trans generational effects. However, the effect of maternal exposure on offspring fitness has not been studied yet.

Globally, the main human exposure routes to BMAA are via the aquatic ecosystem, like through direct contact with phytoplankton infested waters and consumption of BMAA contaminated fish, shellfish and other invertebrates [58]. BMAA has been detected in natural phytoplankton communities (e.g. [83, 102]) and in laboratory cultured cyanobacterial and diatom isolates [94, 98]. Although reported values vary widely, BMAA concentrations in phytoplankton (when present) are expected to lie in the ng/g dry weight (DW) to low μg/g DW range [58]. In addition, BMAA has been found in animals used for human consumption, such as crabs, oysters, mussels and fish (e.g. [83, 100, 145]), in concentrations similar to those for phytoplankton [58].

To test whether trans generational effects of BMAA exposure occur, animals with short generation times can best be used. The water flea *Daphnia magna* reproduces fast and asexually, and is easy to culture. Moreover, *D. magna* is naturally exposed to BMAA, as it can feed on BMAA containing phytoplankton species such as cyanobacteria and diatoms. It may therefore be a vector for BMAA transport along the pelagic food chain, and as such contribute to human BMAA exposure through fish consumption. Indeed, BMAA has been found in natural zooplankton populations at concentrations below 0.1 μg/g DW [83] and in experimentally exposed zooplankton [129]. Finally, herbivore zooplankton species, such as *D. magna*, play an important ecological role in freshwater systems. Under favourable circumstances, like low predation pressure, they can control phytoplankton abundance and contribute to a clear water system [8]. They transfer energy from primary producers to higher trophic levels and are therefore key species in many aquatic food chains [8]. Changes in *Daphnia* fitness may therefore result in altered phytoplankton composition and abundance, as well as in food web changes.

BMAA in dissolved form is not acutely lethal to *D. magna*, but exposure to concentrations of 10 - 100 µg/l increased size at first reproduction and decreased population growth rate in a previous study [129]. Moreover, BMAA was detected in offspring from exposed *D. magna* mothers [129]. We therefore expect that BMAA exposure of *D. magna* mothers might affect their offspring's fitness. As shown for the cyanobacterial toxin microcystin-LR, such trans generational effects can both enhance or decrease offspring fitness. In one study, cyanobacterial extracts and microcystin-LR reduced survival and delayed maturity in offspring from exposed mothers [179]. However, in some cases *Daphnia* can adapt to cyanobacterial exposure, as shown in another study [180]. In case adaptation occurs, offspring from exposed mothers can have an increased fitness when exposed to cyanobacteria compared to offspring from unexposed mothers [181]. This increased fitness might be caused by microcystin-LR induced maternal transfer of detoxification enzyme activation, which results in higher offspring survival under exposed conditions [182]. As shown for *D. carinata*, this maternally transferred inducible tolerance is clone specific, and could come at the expense of reduced fitness under unexposed conditions [183].

Current toxicological studies focus on direct effects of BMAA exposure. Given the expected trans generational effects of BMAA exposure, multi-generation studies are also needed. The aim of our study is to determine whether maternal exposure to BMAA affects offspring fitness in *D. magna*, a key species in aquatic systems that has the potential to transfer BMAA from phytoplankton to human food. We hypothesize that maternal exposure to BMAA negatively affects their offspring's population growth rates and that *D. magna* does not adapt to BMAA exposure. To test these hypotheses, we performed a life history experiment with offspring born from unexposed and exposed mothers, that were raised in either clean or BMAA containing medium. We furthermore performed an uptake experiment to see whether BMAA could be maternally transferred.

6.2 METHODS

6.2.1 Daphnia magna maintenance and pre-culture

Daphnia magna Straus used in our experiments was isolated from the Dutch lake Zwemlust in 1999 and was maintained in the laboratory as described earlier [129]. Both experiments were performed with neonates from a new isofemale lineage [129]. The life history experiment was performed in 2010 and the BMAA uptake experiment was performed in 2015.

6.2.2 Life history experiment

Neonates (less than 24 hours old) were placed individually in glass tubes and were either placed in clean medium (n=12, control treatment, C), or received medium with a BMAA concentration of ~110 µg/l (n=12, BMAA treatment, B). The first two broods of this F_0 generation animals were immediately removed from the tubes. Neonates of the third broods

(generation F_1, less than 24 hours old) from different mothers were individually transferred to new glass tubes. One or two neonates of each mother were transferred to clean medium, another one or two to BMAA containing medium (Figure 6.1). This resulted in the following treatments: transfer from clean medium to clean medium (n=11, control-control treatment, CC), from clean medium to BMAA containing medium (n=11, control-BMAA treatment, CB), from BMAA containing to clean medium (n=9, BMAA-control treatment, BC) and from BMAA containing to BMAA containing medium (n=10, BMAA-BMAA treatment, BB). These F_1 animals were kept until they produced their third brood; all broods (the F_2 generation) were immediately removed from the tubes. Survival, somatic growth, reproduction and population growth of the F_1 generation, and presence of BMAA in the animals from the F_1 and F_2 generation were endpoints for this experiment.
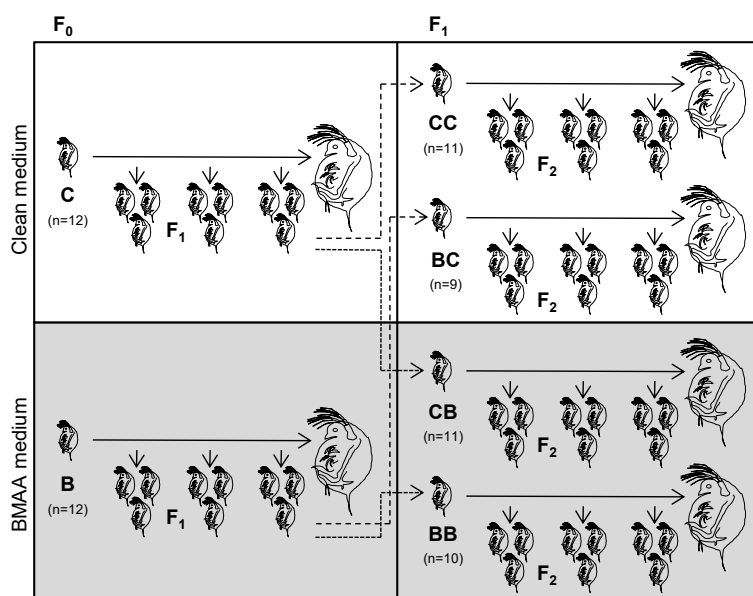

Figure 6.1. Setup of the life history experiment.

All glass tubes used in this experiment contained 100 ml of RT medium [184] and the green algae *Scenedesmus obliquus* SAG 276/3a at ~5 mg C/l. In the treatments where animals were exposed to BMAA, BMAA (L-BMAA hydrochloride, Sigma-Aldrich) was added to the medium at a nominal concentration of ~110 μg/l. BMAA stocks were prepared weekly, and the nominal concentration in the medium ranged between 104 and 117 μg/l due to different stock concentrations. F_0 animals were transferred to new tubes with clean medium, food and BMAA (if appropriate) every two days, F_1 animals were transferred daily. The experiment was performed at 22 °C and a light-dark regime of 18-6 hours at 7.4 μmol photons/m^2 s in an incubator (Gallenkamp) without shaking.

Every two days (F_0 animals), and nearly every day (F_1 animals), survival, number of living and dead neonates and body length were recorded. Body length was measured under a dissecting microscope and was defined as the distance from the most posterior point on the eye to the base of the junction of the tail spine with the carapace. After animals were removed from the experiment (neonates immediately after they were born, adults at the end of the experiment), they were thoroughly rinsed and placed in clean medium for a few hours. Animals were subsequently placed in small aluminium cups for dry weight determination. All adults were pooled per treatment. For neonates from F_0 adults, 3 to 4 broods per treatment (all belonging to the same brood number) were combined. The neonates from F_1 adults were pooled per brood. The animals were dried overnight at 50 °C before weighing on a MC5 microbalance (Sartorius). At the start of the experiment, body length and dry weight of 24 F_0 neonates that were not used in the experiment were determined as a representative of t=0.

6.2.3 BMAA uptake experiment

The setup of the BMAA uptake experiment was similar to that of the life history experiment, except that the animals were not kept individually, but with 20 individuals in one jar to obtain enough biomass for the BMAA analysis. Twenty neonates (less than 24 hours old) were placed in glass jars and were either placed in clean medium (n=3, control treatment, c), or received medium with a nominal BMAA concentration of 78 µg/l (n=3, BMAA treatment, b). The animals were fed daily, and the first two broods of this F_0 generation animals were immediately removed from the jars, as were the dead animals. When the eggs from the third brood of the F_0 animals were deposited in the brood sac, the F_1 mothers were transferred to the new exposure conditions. Half of these animals were transferred to clean medium, the other half to BMAA containing medium. This resulted in similar treatments as in the life history experiment: control-control treatment (cc), control-BMAA treatment (cb), BMAA-control treatment (bc) and BMAA-BMAA treatment (bb, all n=3). After half of the F_0 mothers had produced their third brood, 20 F_1 neonates were randomly selected and were used for the second part of the experiment. The other neonates (F_1) and the adults (F_0) were removed from the jars. In the second part of the experiment, the 20 selected F_1 neonates were kept in the treatment in which they were born, until they produced their third brood. All broods (F_2) were immediately removed from the jars. Survival, total number of offspring, brood mortality and concentration of BMAA in the animals were endpoints for this experiment.

All glass jars used in this experiment contained 800 ml of RT medium. Animals were transferred to new jars with medium and BMAA (if appropriate) at each pregnancy and after giving birth. The medium of the jars in which the animals were kept up to their first pregnancy was refreshed every three days. The animals were fed with *S. obliquus* SAG

276/3a, an amount corresponding to a food concentration of ~5 mg C/l was added daily. The experiment was performed in the dark at 20 °C in an incubator without shaking.

Every day, survival and number of living and dead neonates were recorded. After living animals were removed from the experiment, they were thoroughly rinsed and placed in a beaker containing 100 ml of clean medium with ~5 mg C/l of *S. obliquus* for three hours. After these three hours, they were rinsed again and stored at -20 °C in Eppendorf tubes. Animals were pooled per replicate and brood: for each jar, the adults were pooled, as were the first broods, the second broods and the third broods.

6.2.4 BMAA analysis

The dried *D. magna* samples from the life history experiment were removed from the aluminium cups and were prepared and analysed for BMAA by LC-MS/MS as in [129]. In short, dried samples were hydrolysed in 6 N HCl vapour for 20 hours at 105 °C in a hydrolysis workstation (Eldex). After drying, these samples were reconstituted in 300 μL acetonitrile/water/formic acid (*v/v* 65:35:0.1) and transferred to a vial for analysis. Analysis was performed on an Agilent 1200 LC and 6410 MS/MS. These analyses were performed before we developed our method with internal standard [93] and the BMAA content of the samples from the life history experiment could therefore not be quantified. BMAA concentrations in the medium were not determined, as medium was refreshed daily and BMAA was expected to be stable from previous test results.

Daphnia samples from the uptake experiment were analysed according to [93] with slight modifications. Before analysis, samples from the uptake experiment were lyophilized and homogenized with a plastic stick. Depending on the available amount of sample material, 0.2 up to 1.1 mg (less than 0.2 mg for some brood samples) was transferred to a small glass tube and 40 μl of a 2 mg/l D_3BMAA (D_3BMAA hydrochloride, Novakits) solution in 20 mM HCl was added. After the samples were dried under vacuum, 30 μl 6 N HCl was added to each sample and after flushing with nitrogen gas, liquid hydrolysis was performed for 17.5 hours under vacuum in a hydrolysis workstation at 105 °C. After hydrolysis, samples were dried under vacuum and reconstituted in 1 ml of acetonitrile/water/formic acid (*v/v* 67:33:0.1) and filtered in a tube with a 0.2 μm cellulose acetate filter (Grace Davison Discovery Science) for 5 minutes at 16000 * g. The filtrate was transferred to a vial, stored at 4 °C and analysed by LC-MS/MS within two weeks.

The medium from the uptake experiment was regularly sampled. These samples were filtered over a 0.2 μm cellulose acetate filter for 5 min at 16000 * g. 300 μl of the filtrate was transferred to a vial and 600 μl of acetonitrile with 0.15% formic acid (v:v) was added. The samples were stored at 4 °C awaiting analysis.

LC-MS/MS analysis was performed according to [93] with slight modifications. Separation was performed on an Agilent 1260 LC, with a 2.1 x 150 mm, 5 µm ZIC-HILIC column (SeQuant, Sweden). Column temperature was 40 °C, injection volume 5 µl and flowrate 0.4 ml/min. The mobile phase consisted of acetonitrile with 0.1 % formic acid (*v/v*, eluent A) and water with 0.1% formic acid (*v/v*, eluent B). The elution program was 0–2 min: 95% A, 4 min: 65% A, 8–17 min 55% A, 17–23 min 95% A, the first 4 and last 6 minutes were directed to waste. BMAA, D_3BMAA, α,γ-diaminobutyric acid (DAB, DAB dihydrochloride, Sigma) and *N*-(2-aminoethyl) glycine (AEG, TCI) were detected on an Agilent 6460 MS/MS. Nitrogen was used as drying, sheath and collision gas and source settings were: drying gas temperature 230 °C, drying gas flow 12 l/min, nebulizer pressure 40 psi, sheath gas temperature 250 °C, sheath gas flow 12 l/min, capillary voltage 2500 V, nozzle voltage 500 V. Fragmentor voltage was 50 V and both quadrupoles were operated in unit mode. The ESI source was operated in positive mode, and the following transitions were monitored in MRM for D_3BMAA: *m/z* 122.1 to 105.1 (4 V collision energy), 88.1 (8 V) and 76.2 (8 V). For BMAA, DAB and AEG, the following transitions were monitored: *m/z* 119.1 to 102.1 (BMAA, DAB, AEG, 4 V), 101.1 (DAB, 4 V), 88.1 (BMAA, 8 V), 76.2 (BMAA, 8 V) and 74.2 (DAB, 4 V). For D_3BMAA, the ratio between quantifier *m/z* 105.1 and qualifier *m/z* 88.1 was 27%, the ratio between *m/z* 105.1 and *m/z* 76.2 was 43%. For BMAA the ratios between quantifier *m/z* 102.1 and qualifiers *m/z* 88.1 and 76.2 were 25%. For both compounds, a relative deviation from these ratios of 20% was allowed. DAB and AEG were not quantified in this study, but only included in the analysis to exclude co-elution with BMAA. In the *Daphnia* samples, BMAA was quantified against an external calibration curve and corrected for D_3BMAA recovery. Medium samples were not spiked with D_3BMAA as there was no bias during workup and analysis (recovery 102%, SD 1.6, n=3), these samples were directly quantified against the BMAA calibration curve.

LOD (based on signal to noise (S/N) ratio for all three transitions of at least 3) for BMAA was an injected amount of 34 fmol, LOQ (S/N ratio of the quantifier at least 10, S/N ratio of the two qualifiers at least 3) was 84 fmol. Recovery in *D. magna* samples was 108% (SD 16, n=72). Retention times of BMAA, D_3BMAA (both 11.6 min) and DAB (12.5 min) were similar as in [93], no chromatograms are therefore shown in this manuscript. AEG was baseline separated from DAB at 13.8 min.

6.2.5 Data analysis

In the life history experiment, somatic growth was determined for each adult by fitting the equation $f = (a \cdot b)/(a + (b - a) \cdot \exp(-c \cdot z))$ through its body length data (*z*, mm) in Sigmaplot 12.0. The initial body length (*a*, mm) was constrained at the measured value, *b* (proxy for the maximum body length, mm) and *c* were estimated. *c* was used as an estimation of the maximum growth rate (1/day).

Population growth (r, 1/day) was approached as follows for the first three broods: $r \approx \ln R_0 / T_c$, in which net productive rate (R_0) is defined as $R_0 = \sum_{x=0}^{\Omega} l_x \cdot m_x$ with l_x the probability to survive until age class x (death by handling is excluded from these calculations) and m_x the number of living offspring produced at age class x; and cohort generation time (T_c, day) is defined as $T_c = \sum_{x=0}^{\Omega} x \cdot l_x \cdot m_x / R_0$ [185]. Within treatment variability in r was estimated by the Jackknife technique [186].

Most statistics were performed on untransformed data in Sigmaplot 12.0, except for ANCOVAs, they were performed on untransformed data in SPSS 19. ANCOVAs were used to correct for the F_1 size at birth during the life history experiment, and were only performed if the dependent variable met the criteria for normal distribution and homogeneity of variance, and if it was determined for each surviving individual.

6.3 RESULTS

In this section, the results of the second part of both experiments (F_1 exposure) are discussed. Treatments from the life history experiment are indicated with uppercase abbreviations (i.e. CC, CB, BC and BB), treatments from the uptake experiment are indicated with lower case abbreviations (cc, cb, bc and bb). Life history results of the F_0 exposure for both experiments are shown in Appendices A6.1 and A6.2.

6.3.1 Survival

In the life history experiment, at least 75% of the animals survived for each treatment (Figure 6.2). Survival was highest (100%) in the controls (CC). Survival was approximately 80% in all treatments in which animals were directly or via the F_0 generation exposed to BMAA (CB, BC and BB). When the neonates of treatment B were transferred to treatments BC and BB, 20% of the animals died within a day. After this first day of the F_1 exposure, no further treatment related mortality occurred in treatments BC and BB. In the CB treatment, mortality only occurred in the first five days after transferral. In treatments CB, BC and BB, one animal was killed during handling, but these deaths are not included in the survival calculations.

In the uptake experiment, survival was 88% or higher in treatments cc, bc and bb. In treatment cb however, high mortality occurred at day three (survival percentages of 75%, 55% and 15% in the separate jars, respectively) and incidental mortality occurred at subsequent days, resulting in a 48% overall survival at the end of the experiment (Table 6.1).

6.3.2 Somatic growth

Animals that were directly exposed to BMAA (treatments CB and BB) were smaller at the end of the experiment than animals raised in clean medium (CC and BC, Figure 6.3). Initially, F_1 animals originating from BMAA exposed mothers (BC and BB) were smaller than those
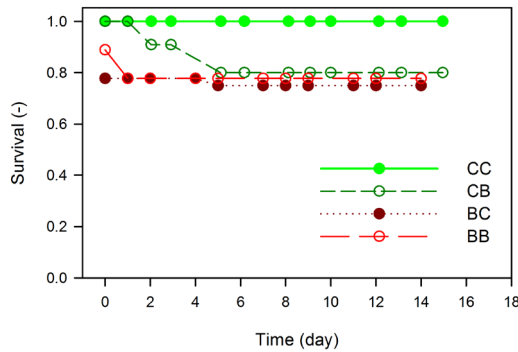
Figure 6.2. Survival of _D. magna_ during the F$_1$ life history experiment. Survival is corrected for death by handling of one animal in treatments CB, BC and BB. n varies from 11 (CC and CB) to 9 (BC).

Table 6.1. Life history parameters of F$_1$ generation _D. magna_ in the uptake experiment. F$_1$ animals from unexposed mothers were either kept in clean medium (cc) or exposed to BMAA (cb), F$_1$ animals from BMAA exposed mothers were treated similarly (bc and bb). SD means standard deviation, homogeneous subsets are indicated with similar symbols and determined by pairwise comparison (Holm-Sidak method, significant at p < 0.05) when treatment effects were significant, n=3 for all treatments.

			cc	cb	bc	bb	Statistical information
Survival	**(%)**	Mean	**88**	**48**	**95**	**91**	Kruskal-Wallis ANOVA
		SD	5.8	31.7	0.2	6.1	$H_3 = 7.89$, p = 0.055
		group	a	a	a	a	
Total neonates brood 1[a]		Mean	**92**	**43**	**145**	**145**	One Way ANOVA
		SD	7.2	32.7	36.3	12.2	$F_{3,8} = 11.25$, **p = 0.003**
		group	a,b	b	a	a	
Total neonates brood 2[a]		Mean	**155**	**84**	**307**	**231**	One Way ANOVA
		SD	20.8	63.7	109.0	104.2	$F_{3,8} = 4.07$, **p = 0.05**
		group	a	a	a	a	
Total neonates brood 3[a]		Mean	**265**	**111**	**323**	**309**	One Way ANOVA
		SD	46.4	53.8	120.7	45.0	$F_{3,8} = 5.22$, **p = 0.027**
		group	a,b	b	a	a,b	
Mortality in broods	**(%)**	Mean	**0.1**	**0.0**	**1.0**	**0.5**	Kruskal-Wallis ANOVA
		SD	0.13	0.00	1.46	0.47	$H_3 = 6.75$, p = 0.080
		group	a	a	a	a	

[a] Only living offspring are considered

from control mothers (CC and CB, Mann-Whitney rank sum test, U = 25.00, n$_C$= 22, n$_B$ =15, p<0.001). However, BC animals had a similar length at the end of the experiment as the animals that were neither directly nor indirectly exposed to BMAA (CC). Likewise, although the CB animals were initially larger than the BB animals, their final length was the same (Figure 6.3, Table 6.2). The differences in growth rate occurred around day 4 (Figure 6.3). The maximum growth rate of CB animals was lower than that of the other animals (Table 6.2). When corrected for size at the start of the experiment, the effects of direct F$_1$ exposure

on final length (ANCOVA, $F_{1,29}=138.28$, $p < 0.001$) and on maximum growth rate (ANCOVA, $F_{1,29}=13.57$, $p = 0.001$) were still significant.
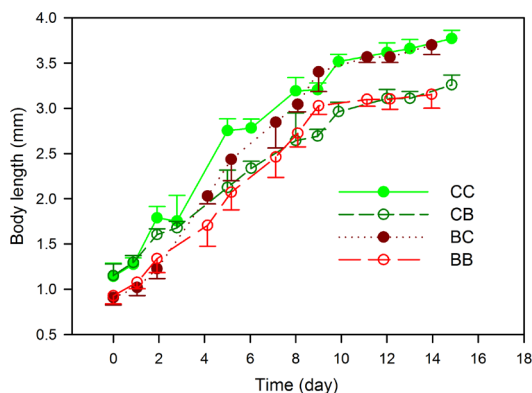

Figure 6.3. Somatic growth of *D. magna* during the F_1 life history experiment. Error bars represent standard deviations, n varies between 6 (BC, end of experiment) and 11 (CC).

6.3.3 Reproduction

In the life history experiment, animals raised in clean medium (treatments CC and BC) produced more living offspring (average 64, SD 12.4, n=17) than animals that were raised in BMAA containing medium (CB and BB, average 32, SD 10.7, n=15, Mann-Whitney rank sum test, U=13.00, $p < 0.001$). When corrected for size at the start of the experiment, the effect of direct F_1 exposure on the number of living neonates in brood 1 was still significant (ANCOVA, $F_{1,29}= 56.43$, $p < 0.001$).The number of living offspring produced was lowest in animals that were exposed to BMAA in the F_0 and F_1 generation (treatment BB, Figure 6.4, Table 6.2). Animals that were only exposed to BMAA in the F_1 generation (treatment CB) also produced less living neonates than animals raised in clean medium, but this was only significant for the first brood. The second and third CB broods belonged both to the statistically defined subgroups of the CC and BC animals, as to the subgroup of the BB animals (Table 6.2).

Furthermore, animals that were directly exposed to BMAA produced dead offspring. In total, 11% of the CB neonates and 32% of the BB neonates were born dead (Table 6.2), and in the third BB brood, the number of neonates born dead was 73% (Figure 6.4).

BMAA exposure, either directly or indirectly, resulted in smaller neonates: BB neonates, for instance, weighed 40% less than CC neonates (Table 6.2). This effect was most pronounced in the third brood (One Way ANOVA, $F_{3,27}=12.76$, $p < 0.001$, Figure 6.5).

Table 6.2. Life history parameters of F$_1$ generation *D.* magna and presence of BMAA in F$_1$ and F$_2$ generations in the life history experiment. In this experiment, F$_1$ animals from unexposed mothers were either kept in clean medium (CC) or exposed to BMAA (CB), F$_1$ animals from BMAA exposed mothers were treated similarly (BC and BB). SD means standard deviation; homogeneous subsets are indicated with similar symbols. d. means detected, n.d. means not detected.

			CC	CB	BC	BB	Statistical information
Survival	**(%)**		**100**	**80**	**75**	**78**	
		n	11	11	9	10	
Final length	**(mm)**	**Mean**	**3.8**	**3.3**	**3.7**	**3.2**	One Way ANOVA
		SD	0.09	0.11	0.11	0.15	$F_{3,28} = 58.02$, **p < 0.001**
		n	11	8	6	7	Pairwise comp. Holm-Sidak
		group	a	b	a	b	significant if **p < 0.05**
Maximum growth rate	**(1/day)**	**Mean**	**0.31**	**0.20**	**0.32**	**0.28**	One Way ANOVA
		SD	0.03	0.03	0.03	0.03	$F_{3,28} = 12.76$, **p < 0.001**
		n	11	8	6	7	Pairwise comp. Holm-Sidak
		group	a	b	a	a	significant if **p < 0.05**
Time of first reproduction	**(day)**	**Mean**	**8.3**	**8.5**	**7.7**	**8.7**	Kruskal-Wallis ANOVA
		SD	0.65	0.67	0.82	0.49	$H_3 = 7.87$, **p = 0.049**
		n	11	8	6	7	Pairwise comparison Dunn
		group	a	a	a	a	all comparisons p > 0.05
Inter-clutch duration	**(day)**	**Mean**	**3.4**	**3.4**	**3.4**	**3.9** [a]	Kruskal-Wallis ANOVA
		SD	0.32	0.32	0.38	0.85	$H_3 = 3.72$, p = 0.293
		n	11	8	6	6	
		group	a	a	a	a	
Size first brood [b]		**Mean**	**16**	**6**	**12**	**9**	One Way ANOVA
		SD	2.2	4.2	3.8	3.7	$F_{3,28} = 13.73$, **p < 0.001**
		n	11	8	6	7	Pairwise comp. Holm-Sidak
		group	a	b	a,c	b,c	significant if **p < 0.05**
Size second brood [b]		**Mean**	**24**	**16**	**26**	**14**	Kruskal-Wallis ANOVA
		SD	6.5	7.0	4.9	8.5	$H_3 = 15.89$, **p = 0.001**
		n	11	8	6	7	Pairwise comparison Dunn
		group	a	a,b	a	b	significant if p **< 0.05**
Size third brood [b]		**Mean**	**25**	**16**	**27**	**3**	Kruskal-Wallis ANOVA
		SD	7.6	1.6	4.0	4.0	$H_3 = 20.14$, **p < 0.001**
		n	11	8	6	6	Pairwise comparison Dunn
		group	a	a,b	a	b	significant if **p < 0.05**
Dry weight neonates	**(µg)**	**Mean**	**6.8**	**5.1**	**4.6**	**4.1**	One Way ANOVA
		SD	2.0	1.4	1.4	1.6	$F_{3,90} = 12.66$, **p < 0.001**
		n	33	24	18	19	Pairwise comp. Holm-Sidak
		group	a	b	b	b	significant if **p < 0.05**
Mortality in broods	**(%)**	**Mean**	**0**	**11**	**0**	**32**	Kruskal-Wallis ANOVA
		SD	0.0	15.7	0.0	22.9	$H_3 = 18.16$, **p < 0.001**
		n	11	8	6	7	Pairwise comparison Dunn
		group	a	a,b	a	b	significant if **p < 0.05**
Population growth rate	**(1/day)**	**Mean**	**0.34**	**0.26**	**0.33**	**0.27**	One Way ANOVA
		SD	0.02	0.05	0.06	0.06	$F_{3, 34} = 6.94$, **p < 0.001**
		n	11	10	8	9	Pairwise comp. Holm-Sidak
		group	a	b	a	b	significant if **p < 0.05**
BMAA in adults (F$_1$)			n.d.	d.	d.	d.	
BMAA in neonates (F$_2$)			n.d.	d.	d.	d.	

[a] One animal only reproduced twice, [b] Only living offspring are considered
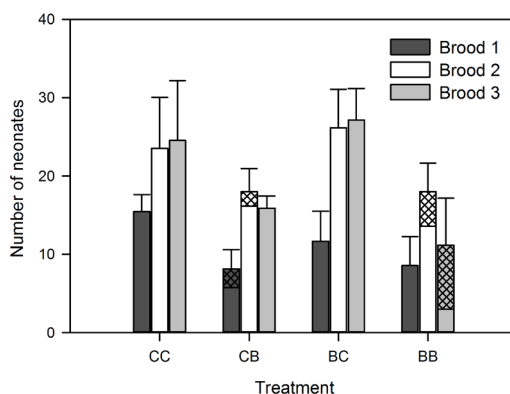
Figure 6.4. Number of living (solid fill) and dead (crossed fill) neonates of *D. magna* during F$_1$ exposure in the life history experiment. Error bars represent the standard deviation of the total number of neonates per brood. n varies between 6 (BC and third brood BB) and 11 (CC).
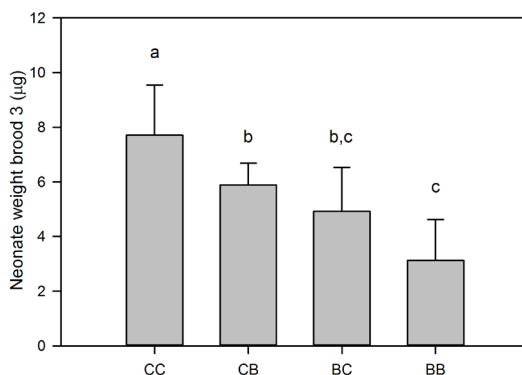
Figure 6.5. Neonate dry weight of the third brood during F$_1$ exposure in the life history experiment. Error bars represent standard deviations, n ranges from 6 (BC and BB) to 11 (CC). Letters indicate homogeneous groups (Holm-Sidak pairwise comparison, significant at p < 0.05).

In the uptake experiment, the cb animals produced the lowest total number of living offspring (Table 6.1). Brood mortality (0.0-1.0%) was similar for all treatments, and was lower than in the life history experiment (Table 6.1).

6.3.4 Population growth rate

Direct BMAA exposure (treatments CB and BB) decreased the population growth rate over 20% compared to the animals raised in clean medium. This decrease was mainly caused by the small number of living offspring produced compared to the F$_1$ animals in clean medium. F$_0$ exposure to BMAA did not affect the fitness of the F$_1$ animals kept in clean medium (treatment BC): despite the higher mortality of BC animals in the first five days, their population growth rate was similar to that of the CC animals (Table 6.2).

6.3.5 BMAA in animals

BMAA was detected in all samples of adults that had directly or indirectly been exposed to BMAA in the life history experiment. No BMAA was detected in the controls (CC adults and neonates). BMAA was also detected in most neonate samples of treatments CB, BC and BB (Table 6.2). Neonate samples in which no BMAA was detected mostly contained little biomass, due to small brood sizes and/or low neonate weight, and therefore likely dropped below the limit of detection.

In the uptake experiment, BMAA was detected in F_0 adults that had been exposed to BMAA either during the first part of their lives (bc), during the last stage of their third pregnancy and directly after giving birth to the third brood (cb) or during their whole lives (bb), although not always in all three replicates (Table 6.3). Furthermore, most of the F_1 neonates exposed to BMAA containing medium for less than 24 hours (cb and bb) contained detectable amounts of BMAA. BMAA was also found in the cb and bb F_1 adults, albeit at lower concentrations than in the F_1 neonates from the same jar (paired t-test, t_4=2.85, p = 0.047). No BMAA was detected in bc F_1 neonates, that were born in clean medium from BMAA exposed mothers. However, in one of the bc jars, BMAA was found in the F_1 adults. Most F_2 neonates born in BMAA containing medium had comparable BMAA concentrations (2.5 up to 5.6 µg/g DW). No BMAA was detected in any of the cc animals.

Table 6.3. Mean BMAA concentrations (µg/g DW) in positive samples, with standard deviations (SD) and number of positive samples (n) in the second part of the uptake experiment. F_0 adults have spent one or two days in the conditions of the second part of the experiment, only to give birth to the F_1 neonates. A part of these F_1 neonates has been removed within 24 hours after birth ('F_1 neonates' in table); the other part was kept under the experimental conditions and was removed from the experiment after giving birth to three broods ('F_1 adults' in the table). F_2 neonates are the offspring of these F_1 adults and were removed within 24 hours after birth. All treatments were performed in triplicate, n.d. means not detected.

	cc			cb			bc			bb		
	mean	SD	n	mean	SD	n	mean	SD	n	mean	SD	n
F_0 adults	n.d.	-	-	45.5	19.88	3	15.37	-	1	8.6	-	2
F_1 neonates	n.d.	-	-	56.2	35.18	3	n.d.	-	-	15.1	-	2
F_2 neonates brood 1	n.d.	-	-	5.6	-	1	n.d.	-	-	5.2	2.04	3
F_2 neonates brood 2	n.d.	-	-	3.5	-	1	n.d.	-	-	4.8	-	1
F_2 neonates brood 3	n.d.	-	-	4.7	-	2	n.d.	-	-	2.5	-	2
F_1 adults	n.d.	-	-	7.8	9.96	3	4.5	-	1	3.4	1.16	3

6.4 DISCUSSION

Below, trans generational effects of BMAA exposure on *D. magna* will be discussed. Most conclusions will be based on the outcomes of the life history experiment, as this experiment was best controlled in terms of food availability, crowding and BMAA exposure. Moreover, as the animals were followed individually, this experiment gives more information on

reproductive output. The uptake experiment will mainly be used to determine uptake characteristics and to assess whether maternal transfer of BMAA occurs.

Direct chronic BMAA exposure (approximately 110 µg/l) decreased *D. magna* maximum growth rate, body length, brood size, neonate weight and clutch viability. Direct exposure caused a 24% reduction of population growth compared to the control animals (compare treatments CB and CC, Table 6.2). This is in line with a previous 15 day exposure study, in which an exposure to 100 µg BMAA/l also reduced population growth rate (calculated over two broods), albeit to a lesser extent (9%, [129]). However, brood mortality and reduced body length were not observed in this earlier study.

Maternal BMAA exposure seemed to have a slightly negative impact on offspring raised in clean medium (compare treatments BC and CC). BC animals had a higher mortality directly after transferral to clean medium, and produced broods with lower weight than the control animals. However, since the BC animals started to reproduce early, and produced as many living offspring as the control animals, their population growth rate was equal to that of the controls. BC neonates were born in BMAA containing medium and spent their first hours (less than 24) in it before they were transferred to clean medium. As *D. magna* can take up BMAA within 24 hours (Table 6.3, [187]), effects of neonate BMAA exposure cannot be ruled out in the life history experiment.

The F_1 generation born from BMAA exposed F_0 mothers did not adapt to BMAA exposure (compare treatments CB and BB). BB animals only performed better than CB animals in terms of maximum somatic growth rate. In contrast, multi-generation exposure negatively affected reproduction in BB animals. Although the population growth rate between CB and BB animals was the same, BB animals produced lighter neonates and had a higher brood mortality. These effects were most pronounced (and significant) for the third brood. *Daphnia* can develop an increased stress tolerance within a lifetime, and transfer this trait to its offspring (e.g. [181, 188]). The duration of the experiment was therefore long enough to demonstrate such rapid adaptation, if our clone would have been able of it. Our clone had been kept in the laboratory for 12 years at the time of the life history experiment, and might therefore have lost some of its ability to deal with cyanobacterial toxins. As adaptation to changing environments is clone specific in *Daphnia*, it is possible that other clones would react differently to multi-generational BMAA exposure. In line with adaptation to the cyanobacterial toxin microcystin, such adaptation would be expected mostly in clones exposed to BMAA containing phytoplankton [180].

In the uptake experiment, measured BMAA concentrations (average 27 µg/l, SD 24.4, n=38) varied and were lower than the nominal concentration of 78 µg/l. This means that under the experimental conditions, BMAA was not stable for a few days. This is in contrast with a

previous test, in which BMAA was stable for 96 hours in a jar containing RT medium but no *Daphnia* and food (20 °C, continuous light, relative SD 6.2%, n=12). The fact that the animals in the uptake experiment were exposed to lower and more variable BMAA concentrations than the animals in the life history experiment (in which medium was refreshed daily), might explain the lack of effect of BMAA exposure on survival, reproduction and brood mortality in the bb animals (Table 6.1) compared to the BB animals (Table 6.2).

The effects of direct BMAA exposure were strongest on juveniles (in both experiments, most treatment related mortality occurred within the first five days and somatic growth reduction was most pronounced before the first pregnancy in BMAA exposed animals, Figure 6.3) and on the number, viability and weight of the offspring (Table 6.2). Once animals started to reproduce, no more adult mortality occurred in the life history experiment, and somatic growth was similar between exposed and non-exposed adults (Figure 6.3). BMAA was detected in the neonates of directly and indirectly exposed mothers (Table 6.2), also in the neonates that were born in clean medium (F_2 BC neonates, Table 6.2 and F_1 bc adults, Table 6.3). This means that BMAA was maternally transferred, and that in *D. magna* females, the internal BMAA concentration is lowered through reproduction. Similarly, in mice, the internal BMAA load in mothers is reduced when BMAA is transferred to their offspring via the placenta and lactation [177, 178].

Unfortunately, we could not determine BMAA concentrations in the life history experiment, our semi-quantitative results were too inaccurate as was shown from mass balances of F_1 neonates and their F_2 offspring. Whether the concentrations determined in our previous *Daphnia* study, using the same method, were accurate cannot be tested [129]. The samples from the uptake experiment were analysed with our improved method, with which BMAA concentrations can accurately be determined.

BMAA was taken up by *D. magna* adults within two days, as the F_0 animals raised in clean medium but transferred to BMAA containing medium only to give birth (cb) contained BMAA (Table 6.3). This is in line with the rapid (within 3 hours) BMAA uptake reported for this species [187]. The BMAA concentration in adults born and raised in BMAA containing medium was 7 times lower at the end of the experiment than when they were neonates, but as the animals increase about 40 times in weight during this period, this decrease is lower than is expected from dilution by growth alone, which indicates BMAA uptake during their lifetime. BMAA losses seemed to occur at a slower speed than initial uptake: some animals that had spent more than 20 (bc F_1 adults) or 14 to 17 days in clean medium (BC F_1 adults) still contained detectable amounts of BMAA.

At present, little is known about BMAA metabolism *in vivo*. In neonatal rats, *in vivo* protein association of BMAA has been suggested, but no BMAA was detected seven months after

exposure [176]. One study on BMAA exposed shellfish did not find evidence for BMAA catabolism, but the majority of the BMAA added to the aquaria could not be retrieved [189]. Similarly, BMAA was taken up by the macrophyte *Ceratophyllum demersum*, but during depuration, free and protein associated BMAA levels in the plant decreased, while there were no indications for catabolism or excretion [190]. This latter study suggests that BMAA was transformed *in vivo* into a form that was undetectable by the analytical methods employed.

Although it is common to either look for 'free' (in an aqueous extract) and 'protein-associated' BMAA (in the pellet created during extraction and released after acid hydrolysis), or for total BMAA (hydrolysis of the total sample), there is a possibility that BMAA is bound to small molecules in the supernatant that are not precipitated during extraction [92]. When only free and protein associated BMAA are analysed, this fraction will be overlooked. We do not know in which forms BMAA was present during our experiment, as we had too little sample material to look for different forms. Instead, we chose to hydrolyse the total sample, which is the safest way of recovering all forms in which BMAA can be present. However, we performed some preliminary tests to determine the fraction in which BMAA is present in exposed *D. magna*. For this, we extracted lyophilized, BMAA exposed *D. magna* with 0.1 N trichloroacetic acid to precipitate proteins, as in [93]. The extract was subsequently dried and hydrolysed in 6 N HCl as described above (section 6.2.4). We found BMAA in this hydrolysed extract, and when compared to the non-hydrolysed extract and total BMAA concentrations, it appeared that approximately 20% of the BMAA was present in the non-hydrolysed extract, and that (most of) the remaining 80% was found in the hydrolysed extract. This agrees with another recent BMAA uptake study in *D. magna*, in which no protein associated, but only free BMAA was detected after 24 hours of exposure (no hydrolysed extract was analysed) [187].

Our study suggests that zooplankton can be an important vector for BMAA transport through the pelagic food chain. BMAA was present in adults and neonates both after direct BMAA exposure and after maternal exposure. This implies that after a BMAA containing phytoplankton bloom has declined, BMAA might still be present in the zooplankton and may from there on be transferred to higher aquatic organisms. Indeed, BMAA has not only been detected in natural zooplankton at concentrations below 0.1 µg/g DW [83], but also in the pelagic fish species Atlantic herring (*Clupea harengus,* 0.01 µg/g DW or lower) that feeds on plankton and is sold for human consumption [83].

Finally, the role of BMAA in phytoplankton is unknown. One possible explanation is that it, like many other non-protein amino acids, acts as a grazer repellent [127, 191]. As shown in this study, BMAA indeed reduced *D. magna* fitness. Our experiment however merely served as a proof of principle, as the dissolved BMAA concentrations were higher than expected in

6

the field: no quantifiable dissolved BMAA concentrations have to our knowledge yet been reported for surface water. While zooplankton grazing can control phytoplankton abundance under certain conditions, cyanobacteria are generally poor food to zooplankton due to their hard-to-handle morphology, low nutritional value and the presence of toxic compounds [8]. As a consequence, cyanobacterial blooms are little constrained by zooplankton grazing. BMAA might be one of the many compounds that protect cyanobacteria from losses by grazing and that contribute to the ongoing expansion and intensification of harmful cyanobacterial blooms. The finding of BMAA in diatoms however questions whether BMAA indeed plays a substantial role in reducing *Daphnia* fitness in field situations: although BMAA has been found in diatoms [98], diatoms generally do not reduce *Daphnia* fitness [192, 193]. Diatoms can reduce fecundity in zooplankton, mainly copepods, but these effects are mostly attributed to oxylipins [193, 194] and not to BMAA. An experiment comparing the fitness of *Daphnia* fed with either non BMAA containing and BMAA containing diatoms could therefore provide more insight in field relevant effects of BMAA on *Daphnia*.

6.5 CONCLUSIONS

Direct BMAA exposure negatively affected *D. magna* fitness: it decreased survival, somatic growth, reproduction and subsequently, population growth rates. Although BMAA is maternally transferred, there was no maternal exposure effect on population growth, as neonates from exposed mothers that were raised in clean medium compensated for initial mortality and lower weight with earlier onset of reproduction. Two generation exposure reduced brood viability and neonate weight, which means that the clone used in this study did not adapt to BMAA exposure. We conclude that in our study, BMAA exposure had trans generational effects, and that these were most pronounced in animals that were exposed to BMAA during two generations. Our results indicate that zooplankton might be an important vector for the transfer of BMAA along the pelagic food chain, but whether BMAA plays a role in preventing zooplankton from controlling cyanobacterial blooms needs further investigation.

ACKNOWLEDGEMENTS

Table A6.1. Life history parameters of F_0 generation *D. magna* kept in clean medium (C) or exposed to BMAA (B) during the life history experiment. SD means standard deviation. d. means detected, n.d. means not detected.

			C	B	Statistical information
Survival	**(%)**		**92**	**83**	
		n	12	12	
Final length	**(mm)**	**Mean**	**3.2**	**2.8**	Independent samples t-test
		SD	0.07	0.15	t_{19} = 7.97, **p < 0.001**
		n	11	10	
Maximum growth rate	**(1/day)**	**Mean**	**0.32**	**0.20**	Independent samples t-test
		SD	0.05	0.06	t_{19} = 5.00, **p < 0.001**
		n	11	10	
Time of first reproduction	**(day)**	**Mean**	**9.6**	**10.4**	Mann-Whitney rank sum test
		SD	0.81	1.58	U = 41.50, p = 0.219
		n	11	10	
Inter-clutch duration	**(day)**	**Mean**	**3.1**	**3.3**[a]	Mann-Whitney rank sum test
		SD	0.30	0.71	U = 28.00, p = 0.083
		n	11	8	
Size first brood [b]		**Mean**	**8**	**6**	Independent samples t-test
		SD	2.4	3.0	t_{19} = 1.42, p = 0.173
		n	11	10	
Size second brood [b]		**Mean**	**10**	**6**	Independent samples t-test
		SD	2.1	3.2	t_{18} = 3.01, **p = 0.007**
		n	11	9	
Size third brood [b]		**Mean**	**7**	**4**	Mann-Whitney rank sum test
		SD	2.4	1.7	U = 13.50, **p = 0.012**
		n	11	8	
Dry weight neonates	**(µg)**	**Mean**	**8.7**	**5.3**	Independent samples t-test
		SD	1.7	1.3	t_{19} = 5.14, **p < 0.001**
		n	11	10	
Mortality in broods	**(%)**	**Mean**	**0**	**7**	Mann-Whitney rank sum test
		SD	0.0	11.5	U = 38.50, p = 0.064
		n	11	10	
Population growth rate	**(1/day)**	**Mean**	**0.25**	**0.19**	Independent samples t-test
		SD	0.03	0.05	t_{22} = 4.06, **p < 0.001**
		n	12	12	
BMAA in adults (F_0)			**n.d.**	**d.**	
BMAA in neonates (F_1)			**n.d.**	**d.**	

[a] One animal only reproduced once, another only twice, [b] Only living offspring are considered

Table A6.2: Life history parameters of F_0 generation *D. magna* kept in clean medium (c) or exposed to BMAA (b) during the uptake experiment. SD means standard deviation, n=3 for all treatments.
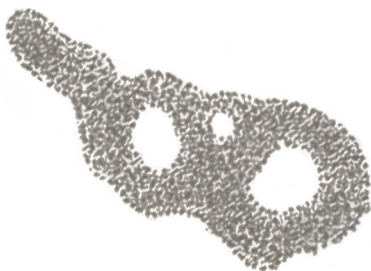
			c	b	Statistical information
Survival	**(%)**	**Mean**	**98**	**61**	Independent samples t-test
		SD	3.2	8.7	$t_4 = 6.95$, **p = 0.002**
Total neonates brood 1[a]		**Mean**	**168**	**75**	Independent samples t-test
		SD	37	29.7	$t_4 = 3.40$, **p = 0.027**
Total neonates brood 2[a]		**Mean**	**199**	**71**	Independent samples t-test
		SD	69.1	23.5	$t_4 = 3.03$, **p = 0.039**
Mortality in broods	**(%)**	**Mean**	**0.5**	**2.1**	Independent samples t-test
		SD	0.22	3.61	$t_4 = -0.74$, p = 0.499

[a] Only living offspring are considered

"Got a mind full of questions and a teacher in my soul
And so it goes"

Eddie Vedder, Guaranteed

CHAPTER 7

A COLLABORATIVE EVALUATION OF LC-MS/MS BASED METHODS FOR BMAA ANALYSIS

ABSTRACT

Exposure to β-*N*-methylamino-ʟ-alanine (BMAA) might be linked to the incidence of amyotrophic lateral sclerosis, Alzheimer's disease and Parkinson's disease. Analytical chemistry plays a crucial role in determining human BMAA exposure and the associated health risk, but the performance of various analytical methods currently employed is rarely compared. A CYANOCOST initiated workshop was organized aimed at training scientists in BMAA analysis, creating mutual understanding and paving the way towards interlaboratory comparison exercises. During this workshop, we tested different methods (extraction followed by derivatisation and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis, or directly followed by LC-MS/MS analysis) for trueness and intermediate precision. We adapted three workup methods for the underivatised analysis of animal, brain and cyanobacterial samples. Based on recovery of the internal standard D_3BMAA, the underivatised methods were accurate (mean recovery 80%) and precise (mean relative standard deviation 10%), except for the cyanobacterium *Leptolyngbya*. However, total BMAA concentrations in the positive controls (cycad seeds) showed higher variation (relative standard deviation 21%–32%), implying that D_3BMAA was not a good indicator for the release of BMAA from bound forms. Significant losses occurred during workup for the derivatised method, resulting in low recovery (<10%). Most BMAA was found in a trichloroacetic acid soluble, bound form and we recommend including this fraction during analysis.

7.1 INTRODUCTION

The neurotoxin β-*N*-methylamino-ʟ-alanine (BMAA) is suspected to play a role in the progressive neurological diseases amyotrophic lateral sclerosis, Alzheimer's disease and Parkinson's disease [37, 46, 59, 69]. Potential routes of human exposure to BMAA include contact with cyanobacteria infested surface waters and ingestion of BMAA containing food, such as fish and shellfish [58]. However, extensive research is needed to determine the precise role of BMAA in the aetiology of these diseases along with characterization of pathways of human exposure.

To assess the health risk associated with BMAA, routes of human exposure are being quantified. BMAA can be present in natural phytoplankton (e.g. [83, 98, 102]) and can be taken up by aquatic organisms such as zooplankton [129, 187, 195], bivalves [189] and macrophytes [196]. Indeed, BMAA has been found in natural zooplankton and shellfish samples [83, 100, 103]. Moreover, it has been detected in other organisms from higher levels of the aquatic food web [83], including fish intended for human consumption [83, 197]. Reported BMAA concentrations in phytoplankton and higher aquatic organisms vary widely, and a substantial part of this variation can be attributed to the use of nonselective analytical methods [93]. BMAA concentrations in aquatic organisms seem to lie within the ng/g dry weight (DW) to µg/g DW range in studies using well described analytical techniques supported by performance data [58].

Analytical procedures (method selectivity and sensitivity, fraction analysed, quality control) play a critical role in assessing the putative link between BMAA and the abovementioned neurodegenerative diseases [75, 85], as well as in the quantification of human exposure pathways [58]. Over the past years, many different analytical methods have been developed and at present, methods using tandem mass spectrometry (MS/MS) detection following proper sample processing are considered most suitable [58, 93, 134]. LC-MS/MS is currently the most frequently applied technique for BMAA analysis and within this technique, diverse sample processing and separation methods are used [58].

In natural samples, BMAA can be present as a free molecule or in bound forms. "Free BMAA" is the fraction obtained by extraction with polar solvents such as 0.1 M trichloroacetic acid (TCA) (Figure 7.1). Bound forms of BMAA can either stay in solution ("soluble bound BMAA") or precipitate during extraction ("precipitated bound BMAA") and BMAA can be released from both bound forms by acid hydrolysis (Figure 7.1). The total BMAA content of a sample is usually obtained by hydrolysis of the total sample (Figure 7.1). The precursor(s) of soluble bound BMAA have not been elucidated yet, but recently it was suggested that in mussels, soluble bound BMAA might not be bound to a peptide or protein [89]. The precursor(s) of the precipitated bound BMAA fraction are also unknown. This fraction is commonly referred to as "protein associated" or "protein bound" [33, 35], but the association of BMAA with

proteins in natural samples still needs to be elucidated. *In vitro*, BMAA can be incorporated into proteins and can be associated to proteins through non-covalent bonding [65, 90], but *in vivo* experiments with bacteria do not show protein incorporation [91].
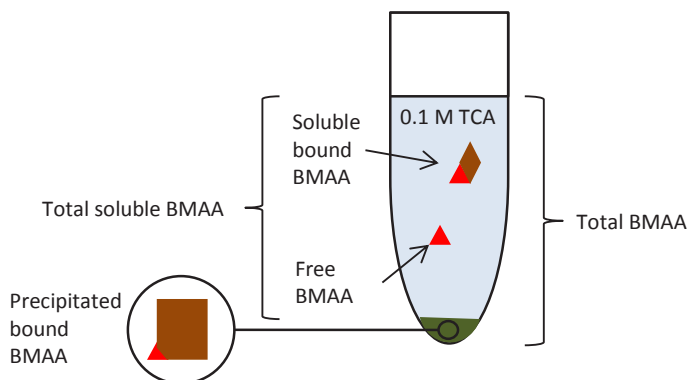

Figure 7.1. Terminology used in this manuscript for the different β-*N*-methylamino-ʟ-alanine (BMAA) fractions. Free and soluble bound BMAA are found in the trichloroacetic acid (TCA) extract. Precipitated bound BMAA is found in the pellet created during extraction. Total BMAA is the sum of all fractions.

Only few studies look at soluble bound BMAA in an isolated fraction (e.g., [89, 92, 103, 198]). In studies where total BMAA (*i.e.*, hydrolysis of the total sample) is (also) determined, ignoring soluble bound BMAA does not lead to an underestimation of the total BMAA concentration. However, when only free and precipitated bound BMAA are analysed (e.g., [33, 36, 102] and more recently [187, 189, 190]) total BMAA concentrations might be underestimated, and the fate of BMAA in experimental systems might be difficult to assess. As an example, in a recent study on BMAA metabolism in the macrophyte *Ceratophyllum demersum*, in which only free and precipitated bound BMAA were analysed, detectable BMAA concentrations in the exposed plants dropped during depuration, while no BMAA was found in the depuration medium and BMAA catabolism did not seem to have occurred. This lead the authors to conclude that BMAA was likely covalently bound in a form undetectable by the analytical methods employed [190].

After extraction, BMAA can be analysed by LC-MS/MS without derivatisation. As BMAA is a small, polar molecule, hydrophilic interaction liquid chromatography (HILIC) is in these cases predominantly used for separation (e.g., [76, 79, 102, 103, 105]). BMAA can also be derivatised after extraction to obtain a larger, more hydrophobic molecule which is easily separated by reversed phase liquid chromatography. Commonly used derivatisation agents are 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC, e.g., [34, 97, 98]), propyl chloroformate (e.g., [91, 99]) and dansyl chloride [100, 101].

As outlined above, analytical chemistry plays an essential role in BMAA risk assessment, but to date, method harmonisation and inter-laboratory comparison of methods have not yet been performed. During a workshop organized in Wageningen University under the auspices of the CYANOCOST network (COST Action ES 1105), analysts from different labs were trained in BMAA analysis and BMAA methods were discussed. By doing so, we aimed to create mutual understanding and to pave the way towards an inter-laboratory comparison exercise and ultimately towards method harmonisation. During this workshop, samples from four relevant matrices (cycad, animal, brain and cyanobacteria) were extracted with at least two different methods (one followed by derivatisation before LC-MS/MS analysis and one directly followed by LC-MS/MS analysis), and each workup was performed by two pairs of analysts. All samples were analysed by LC-MS/MS by one operator. The analysts were experienced in cyanotoxin analysis, were provided with detailed protocols and instructions and were intensively supported by the three trainers who had developed the methods used.

7.2 EXPERIMENTAL DESIGN

Three different sample types, animal samples (seafood and BMAA exposed *Daphnia magna*), brain tissue (unspiked and spiked with BMAA before workup) and cyanobacterial samples (*Leptolyngbya* PCC 73110 and a *Dolichospermum* dominated field sample), were prepared for underivatised and AQC derivatised LC-MS/MS analysis (detailed Materials and Methods are described in Appendices A7.1 (underivatised protocols) and A7.2 (derivatised protocol)). We selected sample preparation methods that were published, validated and developed by the trainers of the workshop (see [93] for underivatised analysis of animal and cyanobacterial samples, [199] for underivatised analysis in brain and [197] for AQC derivatised analysis of all sample types). Where needed, the extraction methods were adapted to the available equipment.

The sample preparations were performed by the workshop participants. An open call was distributed through the CYANOCOST network and the selection of participants was carried out jointly by CYANOCOST Working Group 3: "Cyanotoxin analysis" leaders and by the local organizers. Selection was largely based on the applicants' experience with cyanotoxin analysis, and especially with LC-MS/MS analysis. During the workshop, the following measures were taken to minimize any variation caused by lack of training: Before starting the practical work, all participants attended lectures on the chemical properties of BMAA and on methods of BMAA analysis. All participants were given detailed documented protocols for the different extraction methods and were trained in the techniques and instrumentation used. Constant technical support was provided by three trainers who developed the sample preparation (Ilag/Zguna for protocol D, Combes for protocol B and Faassen for protocol A and C) and by laboratory technicians who had experience with the methods used. All LC-MS/MS analyses were performed on an Agilent 1260 LC coupled to an Agilent 6460 triple quadrupole mass spectrometer by one operator.

The samples that were prepared for underivatised LC-MS/MS analysis were extracted with 0.1 M TCA at ambient temperature to obtain free BMAA. Total BMAA was obtained by 6 M HCl hydrolysis of the total sample. For the animal samples, total soluble BMAA was also determined by hydrolysing the dried 0.1 M TCA extract with 6 M HCl. This fraction was not determined for the other two sample types because we did not have brain and cyanobacterial samples with relatively high BMAA concentrations. The workup for the brain samples included an additional clean-up step by Oasis MCX solid phase extraction (SPE, Figure 7.2).
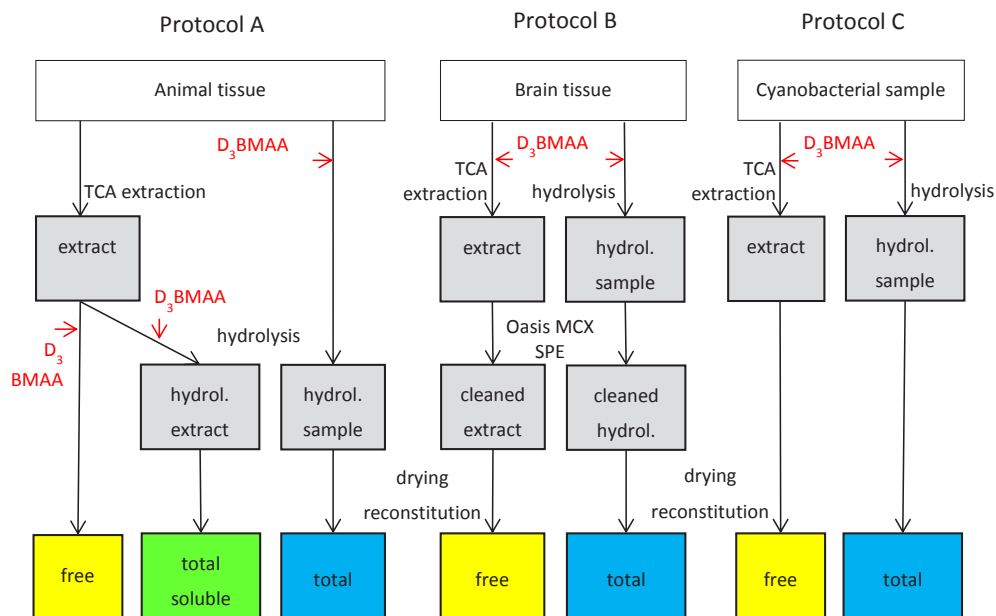
Figure 7.2. Sample preparation schemes for the analysis of underivatised BMAA in three different matrices: animal tissue other than brain (protocol A), brain tissue (protocol B) and cyanobacterial samples (protocol C). The workup for total BMAA is the same in method A and C. Workup for free BMAA in these protocols only differs in the point at which D_3BMAA was added.

In all protocols, D_3BMAA was added as internal standard, and blanks (workup without matrix, negative controls) and cycad seed sarcotesta (positive controls) were included. All samples and controls were prepared in triplicate by two pairs of analyst, resulting in six workups per sample (see Table A7.1.1 in Appendix A7.1).

We intended to use the derivatised protocol for total BMAA determination in all sample types. However, in agreement with a recent method evaluation in an independent laboratory [200], we obtained such a poor recovery with the derivatised protocol (Protocol D, recovery < 10%) that we did not use it for BMAA quantification. From this point on, the manuscript

therefore focuses on the underivatised protocols, and the results and discussion for the derivatised protocol can be found in Appendix A7.2.

7.3 RESULTS AND DISCUSSION

7.3.1 Trueness and precision

Trueness of protocols A, B and C, expressed as mean recovery of D_3BMAA added before workup, were not all within the acceptable range of 70%–120% [201] (Table 7.1). Some fractions of the control samples gave a slightly lower recovery (between 59% and 69%) and D_3BMAA recovery in *Leptolyngbya* was very low (7%–21%). Better recoveries (88%–100% for the free fraction and 56%–75% for the total samples) had previously been obtained for cyanobacterial lab strains extracted with the same protocol [93] and it is unclear what has caused the low recovery in this *Leptolyngbya* strain. In contrast to *Leptolyngbya,* D_3BMAA recovery from the free fraction in *Daphnia* (141%) was too high. When the workup was repeated, D_3BMAA recovery was well within the acceptable range (103%, SD 7.4, *n* = 3).

Table 7.1. Trueness (mean D_3BMAA recovery (%)) and intermediate precision (relative standard deviation of D_3BMAA recovery, *n* = 6, results of both pairs combined), for samples prepared for underivatised analysis. Trueness outside the acceptable range is indicated with blue (<70%) and red (>120%). Precision exceeding the acceptable value (20) is indicated with red [201].

Protocol	Animal (A)			Brain (B)		Cyanobacteria (C)	
Fraction	Free	T.S. [1]	Total	Free	Total	Free	Total
Blank	85 (2.6)	65 (4.9)	81 (13.7)	78 (4.8)	72 (8.4)	100 (7.8)	59 (6.3)
Cycad	93 (7.8)	64 (11.4)	86 (2.1)*	69 (7.5)	73 (2.5)	103 (8.5)	65 (4.3)
Seafood	96 (6.6)	78 (7.9)	108 (6.7)	-	-	-	-
Daphnia magna	141 (2.5)	75 (1.0)	110 (8.0)	-	-	-	-
Brain unspiked	-	-	-	77 (11.1)	84 (15.7)	-	-
Brain spiked	-	-	-	80 (6.0)	82 (9.0)	-	-
Dolichospermum	-	-	-	-	-	103 (7.4)	78 (2.3)
Leptolyngbya	-	-	-	-	-	21 (61.0)	7 (41.5)

[1] Total Soluble, * *n* = 5

Intermediate precision (within-laboratory reproducibility, expressed as relative standard deviation of D_3BMAA recovery) was below 10% for most, and below 20% for all samples except for *Leptolyngbya* (Table 7.1). The workup in protocol A and C was essentially the same for free BMAA and exactly the same for total BMAA, but the extractions were performed on different days. When the results of protocols A and C were combined, the precision was still within the acceptable range: 9.8% for D_3BMAA recovery in the free fraction in blanks, 9.4% in the free fraction of cycads, 19.5% in the total fraction in blanks (all *n* = 12) and 15.1% in the total fraction of cycads (*n* = 11).

In Table 7.1, trueness and intermediate precision were based on the recovery of D_3BMAA that was added as a free compound, as no "bound" D_3BMAA or BMAA is available. When intermediate precision is expressed as the relative standard deviation of the amount of BMAA found in the positive control (cycad seed), which does contain bound forms of BMAA, it shows that in all three protocols, intermediate precision for total BMAA is greater than 20% and that correction for D_3BMAA recovery does not increase precision (Table 7.2). For total BMAA determination, D_3BMAA recovery and the BMAA concentrations uncorrected for D_3BMAA recovery were not correlated (Pearson product moment correlation, p = 0.15, n = 17, see Appendix A7.3), in contrast to the free fraction, where this correlation did exist (correlation coefficient 0.88, p < 0.001, n = 18, see Appendix A7.3). Assuming that the stability of (free) BMAA and D_3BMAA is the same, this implies that during workup for total BMAA (and possibly also for soluble bound BMAA), small procedural variations have affected the release or formation of BMAA, but not, or to a lesser extent, its stability or signal suppression. This also suggests, that although each method seemed precise and accurate based on D_3BMAA recovery, correction for D_3BMAA recovery only results in accurate quantification of free BMAA and not in accurate quantification of bound forms. (Free) D_3BMAA added before sample procession does therefore seem to be a good indicator for losses during extraction and changes in MS/MS signal due to matrix effects, but does not seem to accurately reflect the release or formation of bound BMAA in natural samples.

Table 7.2. Intermediate precision expressed as relative standard deviation of the BMAA concentration (µg/g DW) determined in cycad seed by underivatised analysis, data with and without correction for D_3BMAA recovery are shown (n = 6, results of both pairs combined). Results exceeding the acceptable value (20, [201]) are indicated with red.

Protocol	Animal (A)			Brain (B)		Cyanobacteria (C)	
Fraction	Free	T. S. [1]	Total	Free	Total	Free	Total
uncorrected for D_3BMAA	10.3	8.4	22.9*	13.5	31.4	18.5	20.5
corrected for D_3BMAA	10.4	13.6	23.9*	9.2	31.6	11.6	20.9

[1] Total Soluble, * n = 5

7.4.2 BMAA in blanks and cycad samples

No BMAA was detected in any of the blanks (negative controls). BMAA was detected in the cycad seed (positive controls), free BMAA concentrations averaged 8.8 µg/g DW (SD 1.8, n = 18), which is similar to the value previously determined in the same sample (10.7 µg/g DW, SD 2.9, n = 3 [93]).

BMAA was found in the hydrolysed 0.1 M TCA extract ("total soluble BMAA" in Figure 7.3), and total soluble BMAA exceeded the total BMAA concentration (t-test total soluble *vs.* total BMAA, t_{21} = 3.071, p = 0.006, n = 23, Figure 7.3). Although the average total BMAA concentration in the cycad seed as determined by all three protocols (75.2 µg/g DW, SD

33.1, n = 17) was consistent with previously reported values for this sample (75.0 μg/g DW, SD 10.8, n = 3, [93]), these values differed substantially between the protocols used in this study (Figure 7.3). This implies that the release of BMAA from precursor bound forms, for which the addition of free D_3BMAA as an internal standard does not correct, is sensitive to slight variations in the workup procedure. In our study, hydrolysis was performed overnight and incubation times were not strictly controlled or registered. Although different hydrolysis procedures are currently applied by different labs [134], the effects of variations in hydrolysis conditions have not been systematically evaluated yet. Given the variation observed in the total BMAA determinations our study, this might be worth looking into. This work should be carried out with samples containing bound forms of BMAA, preferably matrix matched certified reference materials. Such materials are not available yet, but the recent finding of BMAA in commercially available mussel material [104] is promising. Until certified reference materials are available, samples that contain a relatively high concentration of bound BMAA, such as cycad seeds, can be used during method development and comparison.
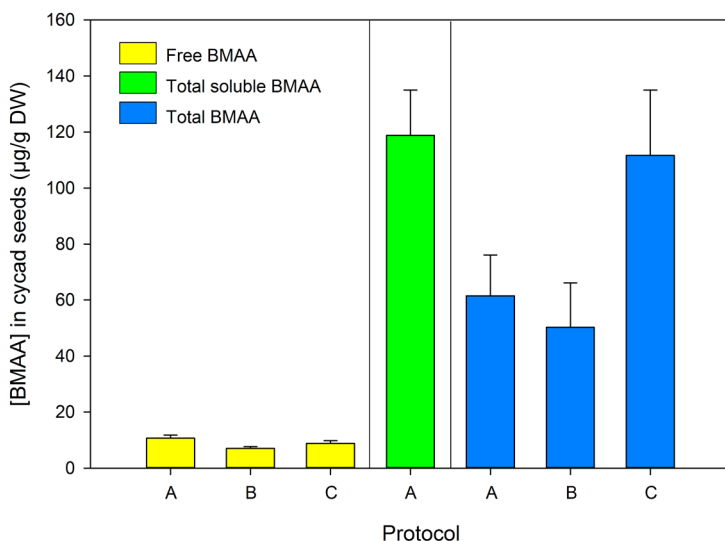
Figure 7.3. BMAA concentrations in cycad seeds as determined by protocols A to C, results for both pairs are combined. Error bars represent standard deviations, n = 6, except for total BMAA determined by protocol A, where n = 5. "Total soluble BMAA" refers to the TCA soluble fraction, including free BMAA.

7.3.3 BMAA in brain tissue

No BMAA was detected in the unspiked brain samples. An additional set of brain samples was therefore spiked with BMAA before sample preparation. After TCA extraction, a BMAA concentration of 3.0 μg/g DW (SD 0.1, n = 6) was determined, which was exactly the spiked concentration. The BMAA concentration determined after hydrolysis of the total sample was 39.9 μg/g DW (SD 3.1, n = 6), which is very close to the spiked concentration of 40 μg/g DW. These findings support our assumption (see Section 7.3.1) that BMAA and D_3BMAA

added before workup (*i.e.* the free compounds) behave similar in terms of stability and signal suppression, both during 0.1 N TCA extraction and during 6 M HCl hydrolysis.

7.3.4 BMAA in animal and cyanobacterial samples

No BMAA was detected in any of the cyanobacterial samples. The *Leptolyngbya* strain used in this study had been shown to contain BMAA at concentrations below 1 µg/g DW with AQC derivatised LC-MS/MS methods [97, 200], but no BMAA was detected in the same strain by underivatised LC-MS/MS analysis ([103], LOD 0.225 µg/g DW). We did not detect BMAA in this strain, but this might be attributed to the high LOD for this sample (estimated at 1 µg/g DW for free BMAA and 20 µg/g DW for total BMAA, as opposed to 0.2 µg/g DW for free BMAA and 2.5 µg/g DW for total BMAA in *Dolichospermum* field samples), which was caused by low recovery in *Leptolyngbya*.

In seafood samples, free BMAA was detected in two replicates, of which one was quantifiable at a concentration of 0.3 µg/g DW. Highest BMAA concentrations were again found in the hydrolysed TCA extract (*t*-test total soluble *vs.* total BMAA, t_{10} = 2.330, p = 0.042, n = 12, Figure 7.4). The variation within each fraction was considerable: relative SD of 21.8 for soluble bound BMAA and 58.2 for total BMAA, where the relative SD of D_3BMAA recovery was below 8% for both fractions (Table 7.1). It is most likely that this variation is caused by small variations during workup (as discussed in Sections 7.3.1 and 7.3.2) and possibly by sample heterogeneity, for both of which the addition of an internal standard cannot correct.
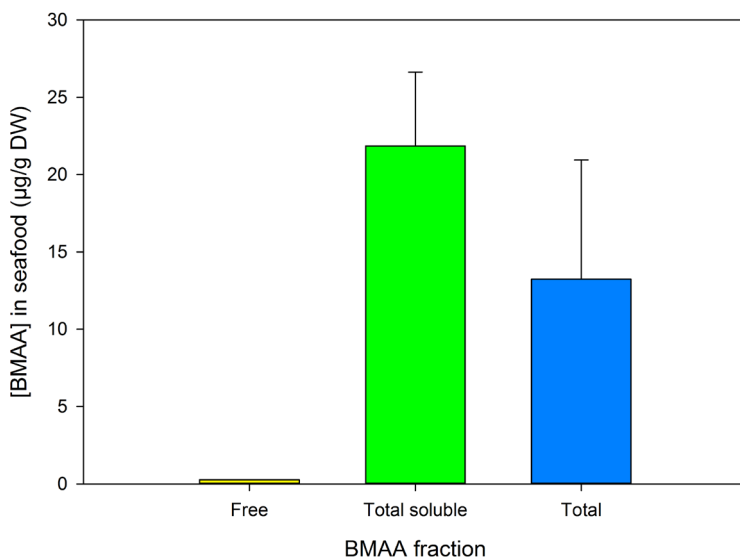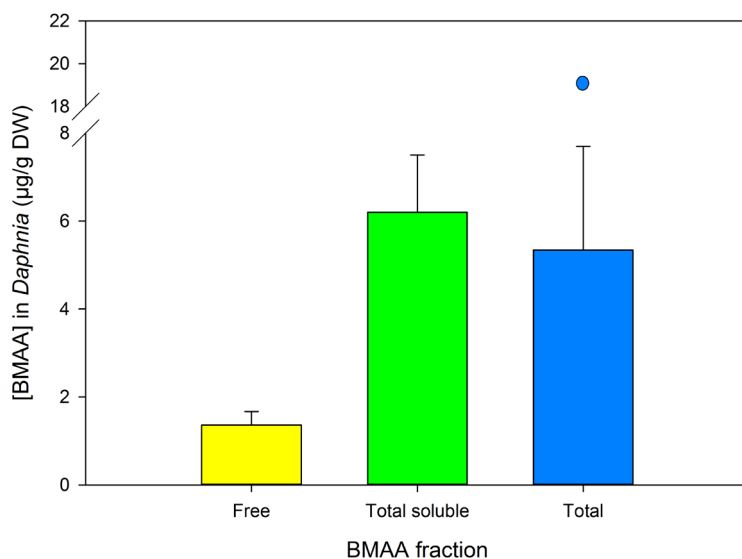

Figure 7.4. BMAA concentrations in seafood samples as determined by protocol A, results for both pairs are combined. Error bars represent standard deviations, n = 1 for free BMAA and n = 6 for each of the other two fractions. "Total soluble" refers to the TCA soluble fraction, including free BMAA.

All *Daphnia* samples contained quantifiable amounts of free and total soluble BMAA (Figure 7.5). Total soluble BMAA concentrations equalled total BMAA concentrations (with outlier included: Mann–Whitney rank sum test, $U = 15$, $p = 0.699$, $n = 12$; without outlier: t-test total soluble *vs.* total BMAA, $t_9 = 0.768$, $p = 0.462$, $n = 11$, Figure 7.5). The variation observed in the total BMAA results may be due to sample heterogeneity along with differences in actual sample size (tissue weight) due to incomplete drying of the animals. Unexposed *Daphnia* and their food source *Scenedesmus obliquus* did not contain detectable amounts of BMAA [93, 195].

Figure 7.5. BMAA concentrations in BMAA exposed *Daphnia* as determined by protocol A, results for both pairs are combined. Error bars represent standard deviations, $n = 6$, except for total BMAA, where $n = 5$ and the sixth data point is presented as outlier. "Total soluble" refers to the TCA soluble fraction, including free BMAA.

7.3.5 BMAA fractions

Free BMAA was found in all cycad and *Daphnia* samples, and in two of the six seafood replicates. Although free BMAA can slowly be released from bound forms during extraction with dilute acid at low temperatures [89], we do not expect that this process has substantially added to the free BMAA concentration we found as our handling times during TCA extraction were short (less than one hour).

In the BMAA positive samples we analysed, total soluble BMAA concentrations (free and soluble bound BMAA, represented by the green bars in Figures 7.3–7.5) were relatively close to the total BMAA concentrations (blue bars in same figures). The tested samples are therefore not expected to contain a high percentage of precipitated bound BMAA. However,

a direct comparative analysis of free, soluble bound and precipitated bound BMAA is needed to definitively answer this question.

The form in which soluble bound BMAA was present in the hydrolysed extract is unclear, because from our experiment we can only derive that it was TCA soluble and that it was bound to a precursor. Whether it is the same low molecular weight, non-protein/peptide precursor as found in mussels [89] is unknown. Further work is needed to identify the structure(s) of this precursor, and to optimize its extraction, as milder methods than the 6 M HCl liquid hydrolysis used in this study have been shown to release soluble bound BMAA in mussels [89].

We detected soluble bound BMAA in all three BMAA positive samples (cycad, seafood and exposed *Daphnia*). Although a limited number of studies have determined this fraction so far, soluble bound BMAA seems to occur in a diversity of organisms: cycad seeds (this study and [92]), periphyton [198], plankton [98, 198], and bivalves [89, 103, 198]. It is therefore recommended to include soluble bound BMAA in future studies, for instance by hydrolysing the total sample (e.g. [93, 100, 197]), or by releasing it from the dried extract [89, 98, 103, 198]. When only free and precipitated bound BMAA are determined, the soluble bound fraction can be overlooked, potentially resulting in a substantial underestimation of the total sample's BMAA content.

7.4 CONCLUSIONS AND OUTLOOK

The three LC-MS/MS based protocols we tested to analyse underivatised BMAA in animal tissue, brain tissue and cyanobacterial samples were generally accurate and precise in terms of D_3BMAA recovery, as well as for free BMAA determination in the positive control (cycad seeds). However, total BMAA determination in cycad seeds was less precise (intermediate precision ranging from 20% to 32%). We suspect that small variations during workup have influenced the liberation or formation of BMAA from bound forms, for which the addition of free D_3BMAA as internal standard could not correct. Given the observed variation in total BMAA concentrations in cycad seeds, we recommend optimization of the workup for soluble bound and total BMAA, which should be performed with samples containing bound BMAA.

The majority of the BMAA detected in the positive samples (cycad seeds, seafood and *Daphnia*) was present in a bound form in the TCA extract. This fraction was released by liquid phase acid hydrolysis, but additional work is needed to identify the structure of its precursor(s) and to optimize its extraction. When only free and precipitated bound BMAA are determined, this fraction will be overlooked. Until its structure has been elucidated and extraction has been optimized, we recommend to include soluble bound BMAA either by determining total BMAA or by hydrolysing (part of) the extract used for free BMAA analysis.

During the workshop, scientists from 12 different research groups were provided with the knowledge and skills to develop appropriate BMAA methods in their own laboratories. Furthermore, mutual understanding was created by an open discussion on the pros and cons of different analytical techniques and by evaluation of the conflicting data in BMAA literature. This common starting point will facilitate the performance of interlaboratory comparison exercises, which are needed to progress BMAA research [58].

ACKNOWLEDGMENTS

7

A7.1.1 Experimental design

Table A7.1.1. Experimental design. Extraction for underivatised LC-MS/MS analysis was carried out by two pairs of analysts (1 to 6) for animal tissue (protocol A), brain tissue (B) and cyanobacterial samples (C). Extraction for derivatised LC-MS/MS analysis was similar for all sample types (D) and was also carried out by two pairs of analysts.

Protocol	A	A	A	B	B	C	C	D
BMAA fraction	free	't.s.'[1]	total	free	total	free	total	total
Blank (neg. control)	5,6	5,6	1,2	1,2	3,4	3,4	5,6	1,3
Cycad seed (pos. control)	5,6	5,6	1,2	1,2	3,4	3,4	5,6	2,4
Seafood	5,6	5,6	1,2	-	-	-	-	3,4
Daphnia magna	5,6	5,6	1,2	-	-	-	-	3,4
Brain unspiked	-	-	-	1,2	3,4	-	-	5,6
Brain spiked	-	-	-	1,2	3,4	-	-	5,6
Dolichospermum	-	-	-	-	-	3,4	5,6	1,2
Leptolyngbya	-	-	-	-	-	3,4	5,6	1,2

[1] t.s.: total soluble BMAA

A7.1.2 Sample origin and storage

The positive control sample consisted of the sarcotesta of *Cycas micronesica* (Hill) seed, which was kindly provided by Chad Husby, Montgomery Botanical Centre, Miami, US. The seed was freeze dried, homogenized by mortar and pestle and stored at −20 °C. The seafood sample was a mixture of crabmeat and Asari clam (*Venerupis philippinarum*), kindly provided by Stephanie Christensen, University of Hawaii, US. The samples were mixed to obtain enough biomass for all analyses. Crabmeat was purchased at a seafood market in Louisiana (US) and shipped to Hawaii frozen on dry ice. It was stored at −80 °C until freeze drying. The freeze dried sample was hand ground with mortar and pestle, and stored in the dark at room temperature. The Asari clam was purchased from a seafood market at Nijiya market, Hawaii, US and prepared and stored the same way as the crabmeat. *Daphnia magna* (Strauss) was isolated from the Dutch lake Zwemlust in 1999, it was maintained in jars containing RT medium [184] and fed with the green algae *Scenedesmus obliquus* SAG 276/3a. Prior to the experiment, the animals were kept under similar conditions, but BMAA (L-BMAA hydrochloride, Sigma) was added to the jars. The animals were exposed to a nominal concentration of 78 μg/L for approximately two weeks. After exposure, animals were rinsed with water, freeze dried and homogenized with a plastic stick. The brain sample was kindly provided by Lonneke IJsseldijk, Utrecht University, The Netherlands and by Mardik Leopold, IMARES, The Netherlands. It was taken from a stranded male harbour porpoise (*Phocoena phocoena,* 33.3 kg, 134 cm), found in Callantsoog (The Netherlands) on 16–06–2008. The animal was still relative fresh (DCC 2), had slightly lost weight (NCC 3) and had probably died of pneumonia. The corpse was kept at −20 °C until dissection and the

whole brain was then stored at the same temperature. Before the start of the experiment, the brain was freeze dried and homogenized in a food processor. The *Dolichospermum* dominated scum sample was collected from a Dutch lake in 2008 and was stored at −20 °C after freeze drying. The lab strain *Leptolyngbya* PCC 73110 was kindly provided by Birgitta Bergman, Stockholm University, Sweden and was grown at 20 °C on BG11 growth medium [165]. After collection by centrifugation and freeze drying, the samples were stored at −20 °C. Samples were prepared and analysed as described in the sections below.

A7.1.3 Protocol A

The protocol used for extraction of animal samples followed by underivatised LC-MS/MS analysis was adapted from a method developed and validated for the underivatised analysis of cyanobacterial samples [93]. Main adaptations are that the total soluble BMAA fraction was included, and that analysis was performed on a more recent LC-MS/MS system, with enhanced sensitivity.

For the extraction of free BMAA and the TCA soluble fraction released by hydrolysis (the 'total soluble fraction'), 8 mg of cycad and 10 mg of *Daphnia* and seafood was used. Samples were extracted by addition of 600 µl 0.1 M TCA, vortexed and left for 10 min at room temperature. Following a further vortex, samples were centrifuged for 5 min at 16000× g and the supernatant was transferred to an Eppendorf tube with a 0.2 µm cellulose acetate filter (Grace Davison Discovery Science, Columbia, USA) and centrifuged for 5 min at 16000× g. TCA (600 µL) was then added to the pellet, and after vortexing, centrifugation and filtration as described above, both extracts were combined. For the analysis of free BMAA, 20 µL of a 2 mg/L D_3BMAA (D_3BMAA hydrochloride, Novakits, Nantes, France) solution was added to 600 µl of the pooled extract. The extract was subsequently dried in a speedvac (SPD121P, Thermo Scientific Savant, Asheville, USA) and reconstituted in 500 µL water/acetonitrile/formic acid (*v/v* 33:67:0.1).

For the analysis of the total soluble fraction, 120 µL of the pooled extract was transferred to a small glass tube, and 40 µL of the 2 mg/L D_3BMAA solution was added. This extract was freeze-dried, and 30 µL 6 M HCl was added to the dry sample. After flushing the sample with nitrogen, it was hydrolysed overnight under vacuum at 105 °C in a hydrolysis workstation (Eldex). After hydrolysis, the samples were dried under vacuum, dissolved in 1000 µL water/acetonitrile/formic acid (*v/v* 33:67:0.1) and filtrated over a 0.2 µm cellulose acetate filter.

Total BMAA was determined in 0.8 mg of cycad seeds and 1 mg of the *Daphnia* and seafood samples. An aliquot of the same D_3BMAA solution (40 µL) was added and the sample was dried under vacuum. Samples were hydrolysed by addition of 6 M HCl (30 µL) and reconstituted as described above for the total soluble fraction.

A7.1.4 Protocol B

The protocol used for brain samples followed by underivatised LC-MS/MS analysis was adapted from a method developed and validated for the underivatised analysis of free BMAA in brain tissue [199]. Main adaptations to this published method are that we included a method for total BMAA, that we started with freeze dried samples instead of with samples that were only frozen and thawed and that we therefore adapted the first extraction steps for free BMAA, and that the LC-MS/MS analysis was performed according to [195] on the same LC-MS/MS system as used for the other analyses in this experiment.

Free BMAA was determined in 8 mg of cycad seed, and 20 mg of harbour porpoise brain. Each pair analysed three unspiked brain samples, three other samples were spiked with 60 ng L-BMAA directly after weighing. An aliquot (40 μL) of the 2 mg/L D_3BMAA solution and 3 mL 0.1 M TCA were added, and the samples were vortexed. BMAA was extracted in an ultrasonic bath (Branson 3510, Danbury, USA) at room temperature for 10 min, after which the sample tubes were centrifuged for 10 min at 3500× g. The supernatant was transferred to a clean glass tube, and solid phase extraction (SPE) was performed using MCX, 60 mg, 3 mL cartridges (Oasis, Etten-Leur, The Netherlands). Cartridges were conditioned with 2 mL of methanol, followed by 1 mL of water with formic acid (pH = 3). Sample (3 mL) was then loaded onto the cartridges, which were subsequently washed with 1 mL cyclohexane. After drying the cartridges with nitrogen gas, 1 mL 0.1 M HCl and 2 mL methanol were added. The samples were then eluted with 3 mL methanol with NH_4OH (freshly prepared by adding 6.6% of a 25% NH_4OH solution to 93.4% of methanol (*v/v*)). After drying in the speedvac, the samples were reconstituted in 1000 μL of water/acetonitrile/formic acid and filtered as described above.

Total BMAA was determined in 0.8 mg of cycad seeds and in 1 mg of brain samples. Directly after weighing, three replicate brain samples for each pair were spiked with 40 ng L-BMAA. An aliquot (40 μL) of the 2 mg/L D_3BMAA solution was added to all samples, and after drying, hydrolysis was performed as described in Section A7.1.3. After hydrolysis, dried samples were quantitatively transferred to new tubes using 0.1 M TCA, final volume was 3 mL. Samples were subsequently cleaned up by SPE and reconstituted as described above.

A7.1.5 Protocol C

The protocol used for cyanobacterial samples followed by underivatised LC-MS/MS analysis was described and validated previously [93], but for this experiment, a more updated LC-MS/MS system was used [195].

Free BMAA was determined in 4 mg of cycad seeds and 5 mg of cyanobacterial samples. To each sample, 20 μL of a 2 mg/L D_3BMAA solution was added. Samples were extracted as described for the animal samples, but only 300 μL of 0.1 M TCA was used during both

extraction steps, instead of 600 μL. After extraction and filtration, the complete extract was dried in a speedvac, and reconstituted in 500 μL water/acetonitrile/formic acid (*v/v* 33:67:0.1).

Total BMAA determination was the same as described for the animal samples (protocol A, Section A7.1.3), the amount of cycad samples used was 0.8 mg, and 1 mg was used for the cyanobacterial samples.

A7.1.6 LC-MS/MS analysis

LC-MS/MS analyses were performed on an Agilent 1260 LC coupled to an Agilent 6460 triple quadrupole mass spectrometer. The method for underivatised analysis is described in [195]. Separation was performed with a 2.1 × 150 mm, 5 μm ZIC-HILIC column (SeQuant, Solna, Sweden). Column temperature was 40 °C, injection volume 5 μL and flowrate 0.4 mL/min. The mobile phase consisted of acetonitrile with 0.1 % formic acid (*v/v*, eluent A) and water with 0.1% formic acid (*v/v*, eluent B). The elution program was 0–2 min: 95% A, 4 min: 65% A, 8–17 min 55% A, 17–23 min 95% A, with linear decreases between the steps. During the first 4 and last 6 minutes the flow was directed to waste. Nitrogen was used as drying, sheath and collision gas and source settings were: drying gas temperature 230 °C, drying gas flow 12 L/min, nebulizer pressure 40 psi, sheath gas temperature 250 °C, sheath gas flow 12 L/min, capillary voltage 2500 V, nozzle voltage 500 V. Both quadrupoles were operated in unit mode and the ESI source was operated in positive mode. MS/MS settings, and precursor to product ion transitions monitored in multiple reaction monitoring (MRM) for BMAA, D_3BMAA, α,γ-diaminobutyric acid (DAB, DAB dihydrochloride, Sigma, Zwijndrecht, The Netherlands) and *N*-(2-aminoethyl) glycine (AEG, TCI) are shown in Table A7.1.2.

Table A7.1.2. MS/MS settings and MRM transitions for underivatised analysis.

Compound	Precursor *m/z*	F[1] V	Quant[2] *m/z*	CE[3] V	Qual[4] *m/z*	CE V	Ratio[5] %	Qual *m/z*	CE V	Ratio %
D_3BMAA	122.1	50	105.1	4	88.1	8	27	76.2	8	43
BMAA	119.1	50	102.1	4	88.1	8	25	76.2	8	25
DAB	119.1	50	101.1	4	74.2	4	-	-	-	-
AEG	119.1	50	102.1	4	-	-	-	-	-	-

[1] Fragmentor voltage, [2] Quantifier ion, [3] Collision energy, [4] Qualifier ion, [5] Ratio between areas of qualifier and quantifier ion

BMAA was identified based on retention time compared to D_3BMAA in the same sample, and by the ratios between quantifier and qualifiers which had to be within a 20% relative range of the same ratios in the calibration standards. DAB and AEG were not quantified in this study, but only included in the analysis to ensure that there was no co-elution with BMAA (Figure A7.1.1). BMAA was quantified against an external calibration curve and each sample was corrected for D_3BMAA recovery. LOD (based on signal to noise (S/N) ratio for all three transitions of at least 3 in a calibration standard) for BMAA was an injected amount of

84 fmol (2 μg/L), LOQ (S/N ratio of the quantifier at least 10, S/N ratio of the two qualifiers at least 3) was 208 fmol (5 μg/L). Response was linear (R^2 = 0.995) within the concentration range of 5 μg/L to 100 μg/L.
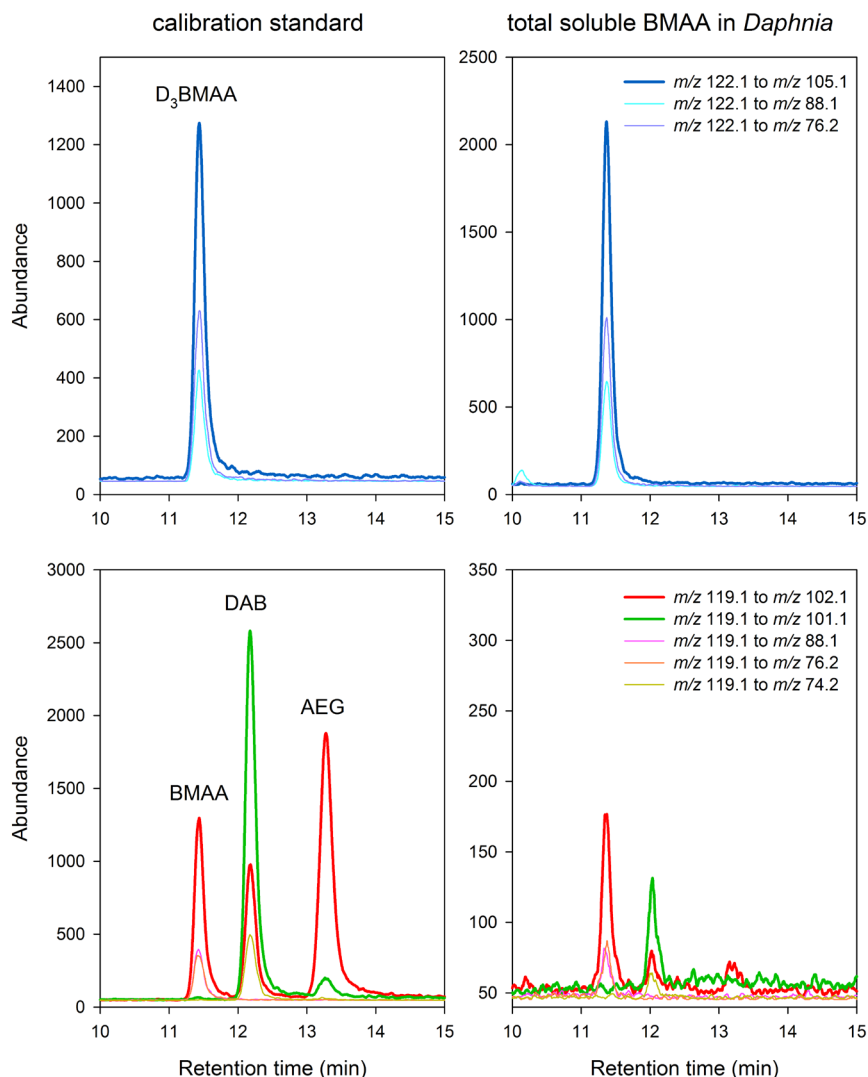

Figure A7.1.1. Chromatograms of underivatised LC-MS/MS analysis of a calibration standard (left panels) and the total soluble BMAA fraction of a *Daphnia* sample (right panels). Quantifiers are indicated by bold lines, qualifiers are indicated by normal lines. The calibration standard contains 50 μg/L of D$_3$BMAA, BMAA, DAB and AEG.

A7.2.1 Main steps workup

The protocol used for derivatised LC-MS/MS analysis was adapted from a previously published and validated method [197]. This protocol was used on all sample types for the determination of total BMAA. The samples were extracted with aqueous methanol, hydrolysed, cleaned up by chloroform extraction and Isolute HCX-3 SPE, derivatised and concentrated before LC-MS/MS analysis (Figure A7.2.1).
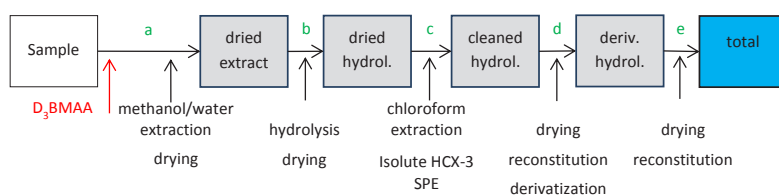

Figure A7.2.1. Sample preparation scheme for the analysis of derivatised BMAA (protocol D). The main steps are indicated by letters a to e.

A7.2.2 Detailed protocol

Total BMAA was determined in 0.5 mg of cycad seed, *Daphnia* and seafood sample, 2 mg of cyanobacterial and brain samples was used. Directly after weighing, 80 ng of L-BMAA was added to half of the brain samples. To all samples, 6 µL of a 100 µg/L D_3BMAA solution was added. Samples were subsequently extracted for 10 min in 500 µL 80% methanol in water (*v/v*) in an ultrasonic bath at room temperature. The samples were then dried down in a speedvac, and transferred to glass vials using 150 µL of 6 M HCl. The vials were closed and the samples were hydrolysed overnight in an oven at 110 °C. After hydrolysis, 300 µL of water was added to the vials, and the samples were filtered for 5 min at 16000× g over a 0.2 µm cellulose acetate filter. After drying in a speedvac, the samples were reconstituted in 550 µL water, and 1000 µL of chloroform was added. After manually shaking for 5 min, the samples were centrifuged for 3 min at 16000× g and 500 µL of the aqueous layer was transferred to a new Eppendorf tube. Water plus 0.2% formic acid (500 µL) was added and the samples were cleaned up by SPE. HCX-3 cartridges (100 mg, Isolute, Uppsala, Sweden) were conditioned with 1 mL of methanol and 1 mL of water with 0.1% formic acid. Samples (1 mL) were loaded, washed with 1 mL of water plus 0.1% formic acid (pH 2.7) and 1 mL of methanol. Samples were eluted by the addition of 2× 800 µL of NH_4OH in methanol (prepared by adding 2.6% of a 25% NH_4OH solution to 97.4% of methanol (*v/v*)). The eluates were dried in a speedvac and reconstituted in 20 µL 20mM HCl. Samples were then derivatised by adding 60 µL of borate buffer and 20 µL of AQC (Waters) derivatisation reagent. The derivatised samples

were again dried in a speedvac, and reconstituted in 30 µL of a water/acetonitrile solution (95:5 *v/v*). Samples were stored at −20 °C before analysis.

As the UPLC method used for the analysis of derivatised samples [197] could not be reproduced on our LC system, we adapted the method for derivatised BMAA analysis as described in [93] to the used LC-MS/MS system to ensure separation of AEG from DAB and BMAA. Chromatography was performed on a Zorbax Eclipse AAA 4.6 × 75 mm, 3.5 µm column (Agilent, Waldbronn, Germany) with the same mobile phases as for the underivatised analysis. The following gradient was applied: 0 min 1% A; 4 min 2% A; 8 min 5% A; 24 min 10% A; 26–30 min 50% A; 30–42 min 1% A with linear increases between the steps. During the first 6 and the last 12 min of each run, the flow was directed to waste. Flow rate was 1 mL/min, injection volume 10 µL and column temperature 40 °C. Source settings were: drying gas temperature 300 °C, drying gas flow 5 L/min, nebulizer pressure 45 psi, sheath gas temperature 400 °C, sheath gas flow 11 L/min, capillary voltage 2500 V, nozzle voltage 500 V. Both quadrupoles were operated in unit mode, and the ESI source was operated in positive mode. MS/MS settings and transitions monitored in MRM for D_3BMAA, BMAA, DAB and AEG are shown in Table A7.2.1.

Table A7.2.1. MS/MS settings and MRM transitions for derivatised analysis.

Compound	Precursor	F[1]	Quant[2]	CE[3]	Qual[4]	CE	Ratio[5]	Qual	CE	Ratio
	m/z	V	*m/z*	V	*m/z*	V	%	*m/z*	V	%
D_3BMAA	462	134	171	35	145	16	12	122	16	27
BMAA	459	143	171	32	258	25	3	119	16	28
DAB	459	134	171	32	315	12	-	-	-	-
AEG	459	134	171	32	214	35	-	-	-	-

[1] Fragmentor voltage, [2] Quantifier ion, [3] Collision energy, [4] Qualifier ion, [5] Ratio between areas of qualifier and quantifier ion

BMAA was identified based on retention time compared to D_3BMAA in the same sample, and by the ratios between quantifier and qualifiers which had to be within a 20% relative range of the same ratios in the calibration standards. DAB and AEG were not quantified in this study, but only included in the analysis to ensure that there was no co-elution with BMAA (Figure A7.2.2). BMAA was quantified against an external calibration curve and each sample was corrected for D_3BMAA recovery. Calibration standards were prepared in 20mM HCl, derivatised in the same way as the samples, and subsequently dried down and dissolved in water/acetonitrile as described above. For BMAA, LOD and LOQ were similar: an amount of 45 fmol on column (corresponding to a concentration of 1 µg/L before derivatisation and concentration). Response was linear (R^2 = 0.995) within the concentration range of 1 µg/L to 100 µg/L.

A7.2.3 D$_3$BMAA recovery

D$_3$BMAA recovery was below 10% in all samples analysed with protocol D which is consistent with a recent independent evaluation of a similar protocol [200]. The majority of the analyte is lost during SPE: when during method development a D$_3$BMAA solution was subjected to SPE, without matrix or previous extraction, and the eluate was derivatised directly after drying (step c and d in Figure A7.2.1), recovery was 37% (SD 7.9, n = 3). When this test was repeated with inclusion of the final concentration step (step e in Figure A7.2.1), no extra losses occurred (recovery 34%, SD 9.0, n=3). In addition to losses during SPE, strong signal suppression has been reported for this method [200]. Because of the low recovery achieved, it was not possible to determine BMAA concentrations in samples using this protocol.

A recovery of 63.3% has been reported for a similar method where the final concentration step was omitted, combined with alternative chromatography and MS/MS detection [135]. However, this value does not cover full recovery, as it is based on the ratio between a spike added before workup and a spike added before derivatisation. Losses that occur during and after derivatisation (like signal suppression) were therefore not taken into account. As the optimum sample protein to derivatisation reagent ratio (0.005 to 0.25 µg protein/µL before derivatisation [124]) is greatly exceeded in this method, it is necessary to determine the efficiency of derivatisation.

The D$_3$BMAA recovery of the protocol used in our study was low, but this is not inherent to AQC based LC-MS/MS methods as in our case, a substantial part of the D$_3$BMAA got lost during workup. If coupled to appropriate workup protocols, AQC based methods can give good recoveries, values ranging from 61% to 99% have been reported for AQC derivatised LC-MS/MS analysis of BMAA (e.g. [93, 145, 149]).
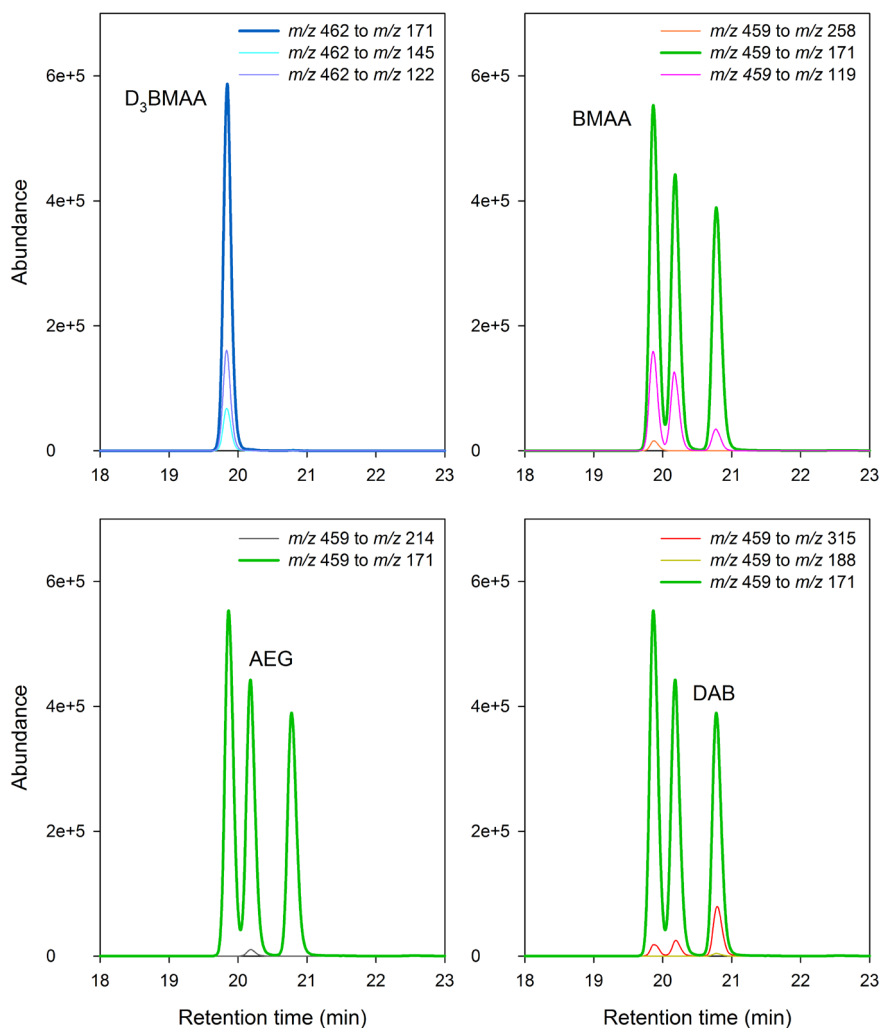
7

Figure A7.2.2. Chromatogram of derivatised LC-MS/MS analysis of a calibration standard containing 500 µg/L of D₃BMAA, BMAA, DAB and AEG. Quantifiers for D$_3$BMAA and BMAA are indicated by bold lines, qualifiers are indicated by normal lines.
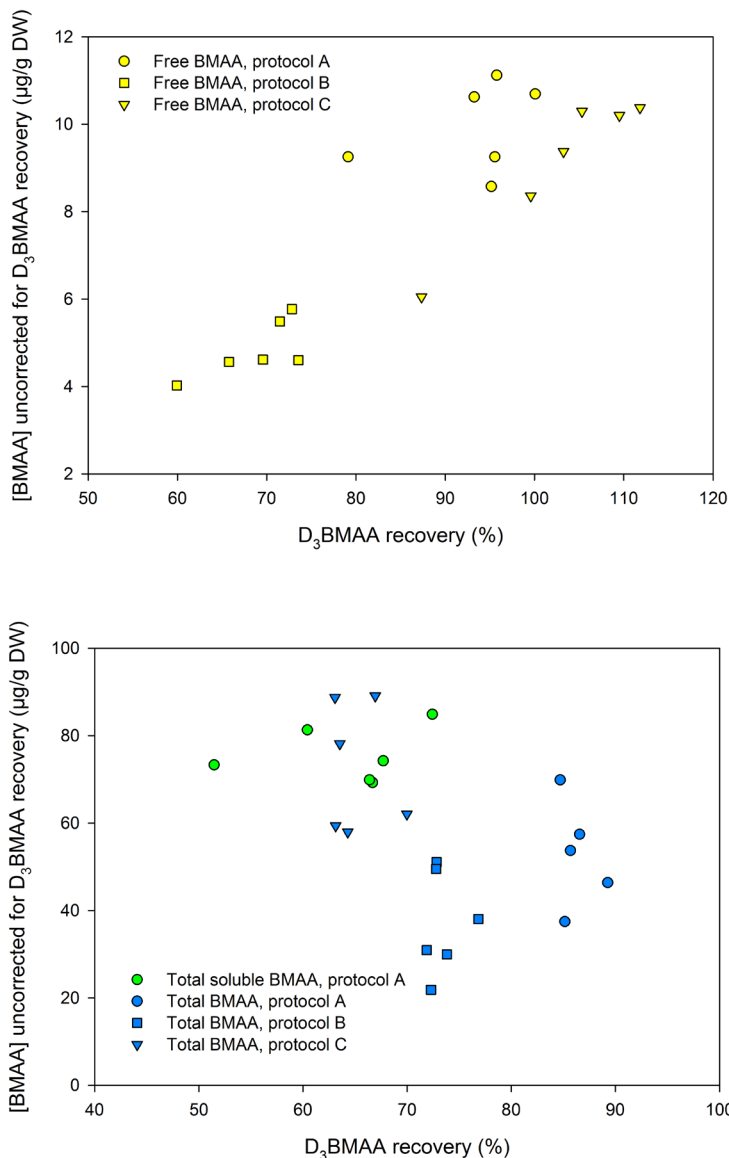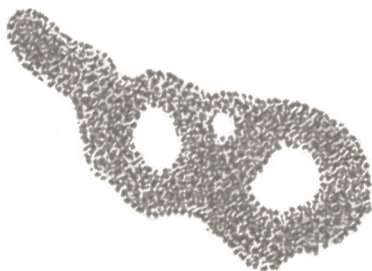
7

Figure A7.3. Relation between D$_3$BMAA recovery and the BMAA concentration determined in cycad seeds by underivatised LC-MS/MS analysis, uncorrected for D$_3$BMAA recovery. The upper panel shows free BMAA, the lower panel shows total soluble BMAA and total BMAA.
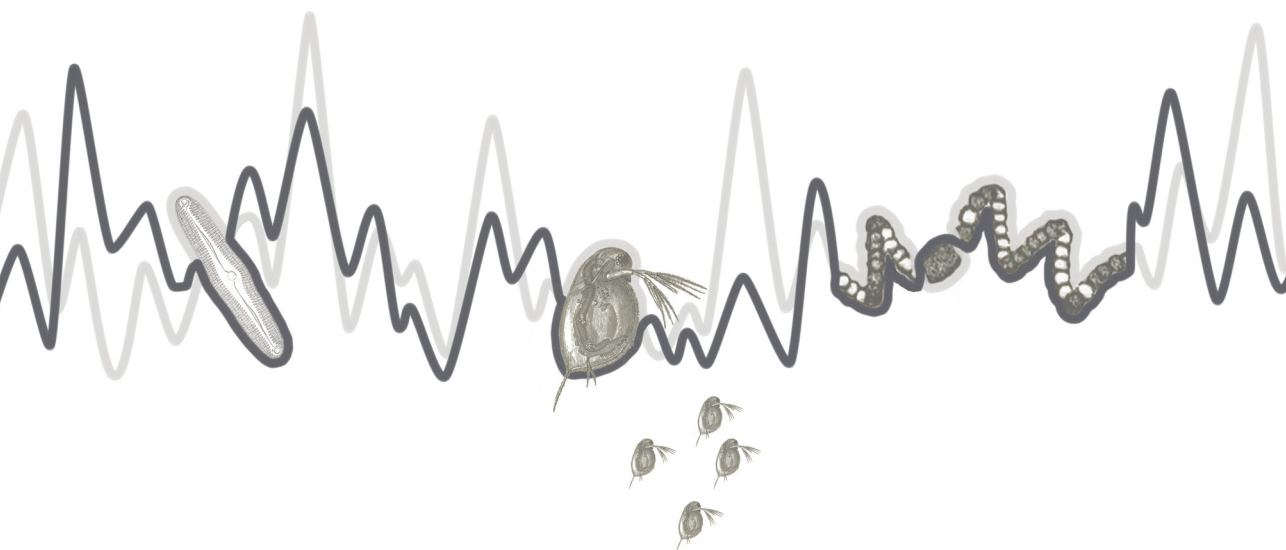
"Ah, (it's) all about chemistry
Won't you show me everything you know?"

Semisonic, Chemistry

CHAPTER 8

Standard operating procedure: Extraction and LC-MS/MS analysis of underivatised BMAA

8.1 INTRODUCTION

Liquid chromatography coupled to tandem mass-spectrometry detection (LC-MS/MS) is a suitable, commonly used technique for the quantification of the neurotoxin β-*N*-methylamino-L-alanine (BMAA) in cyanobacteria [58]. BMAA can either be derivatised before analysis, for instance with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [34, 97], propyl chloroformate [99] and dansyl chloride [100], or it can be analysed without pre-column derivatisation [76, 102].

In LC-MS/MS analysis, BMAA should be properly separated from isobaric compounds such as α,γ-diaminobutyric acid (DAB), *N*-(2-aminoethyl) glycine (AEG) and β-amino-*N*-methylalanine (BAMA) [97, 104]. Furthermore, attention should be paid to the form in which BMAA can be present in natural samples: either free, bound in a fraction that precipitates with proteins or bound in a (0.1 M trichloroacetic acid, TCA) soluble fraction.

The extraction methods used in this SOP (0.1 M TCA extraction and liquid 6 M HCl hydrolysis) are commonly used in BMAA research. However, it is possible that method details like incubation times and conditions will still be optimized. Furthermore, the precursors of the bound BMAA fractions are still unknown and therefore, more effective extraction methods for bound BMAA could be developed [89]. It is therefore recommended to check recent literature before setting up an analytical method for BMAA.

8.2 EXPERIMENTAL

8.2.1 Materials

- Ultrapure water, e.g. with a Qpod (Millipore)
- Acetonitrile at least of HPLC grade
- Hydrochloric acid, trichloroacetic acid and formic acid of analytical grade
- Teflon forceps
- Glass Pasteur pipettes
- 2 mL plastic tubes (e.g. from Eppendorf)
- 2 mL spin filter tubes with 0.2 μm cellulose acetate filter
- Small glass tubes for hydrolysis (6 x 50 mm, when hydrolysis workstation is used)
- HPLC/UHPLC glass vials
- Calibration standards of BMAA, DAB, AEG and deuterium labelled BMAA as an internal standard (D$_3$BMAA), all standards are commercially available.

8.2.2 Special equipment

- Freeze-dryer
- Speedvac
- Microcentrifuge

- Hydrolysis workstation (e.g. Eldex), alternatively, samples can be hydrolysed in a stove, in glass vials with screw caps (in which case samples are analysed under atmospheric conditions, the steps describing nitrogen flushing and application of vacuum can be skipped).
- HPLC or UPLC coupled to a triple quadrupole mass spectrometer
- Integration software

8.2.3 Solutions

- 1 M trichloroacetic acid (TCA)
- 6 M hydrochloric acid (HCl)
- 20 mM HCl
- 67% acetonitrile/33% water/0.1% formic acid solution (v/v/v)
- D_3BMAA spike solution in 20 mM HCl. The concentration of the spike solution and the volume of spiking solution added during sample processing depend on the method LOD. In this SOP, a concentration of 1 μg/ml is used. When the spiking volumes described in section 8.2.5 are used, this results in a concentration of 40 ng/ml in the solution to be analysed (if 100% recovery would be achieved).

8.2.4. LC-mobile phase

- Mobile phase A: Acetonitrile with 0.1% formic acid (v/v)
- Mobile phase B: Water with 0.1% formic acid (v/v)

8.2.5 General procedure

In sections 8.2.5.1 and 8.2.5.2, the extraction of free BMAA, total 0.1 M TCA-dissolved BMAA (including the soluble bound fraction) and total BMAA in cyanobacterial samples is described step to step. The amount of bound BMAA present in the precipitated fraction can be determined by subtracting the amounts of free and dissolved bound BMAA from the total amount in the sample. But preferably, this fraction is directly determined, as described in section 8.2.5.3 (see Figure 8.1).

For all extraction methods, it is recommended to include blank samples (no matrix) and positive controls (e.g. cycad seeds, *Cycas micronesica* (Hill)). Hydrolysis of sample material collected on GF/C filters with the methods described below results in very low recovery, cyanobacterial samples are therefore better concentrated in other ways, e.g. by centrifugation. Furthermore, all extractions should be performed under acidic conditions to ensure BMAA stability.
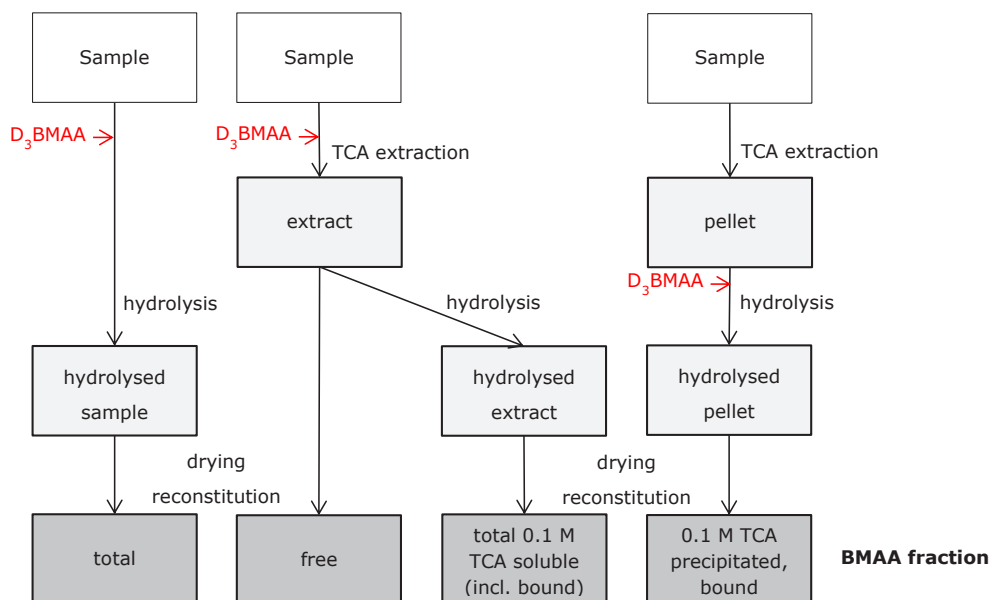
Figure 8.1. Sample preparation scheme for the extraction of different BMAA fractions.

8.2.5.1 Total BMAA

a) Freeze dry samples to disrupt the cell walls.

b) Weigh 1 mg into a small glass tube.

c) Add D_3BMAA spike (40 µL).

d) Dry the sample under vacuum in the hydrolysis workstation.

e) Add 30 µL 6 M HCl to each glass tube. After HCl addition, use Teflon forceps to handle the tubes.

f) Flush sample three times with nitrogen in the hydrolysis workstation, apply vacuum between flushing steps.

g) Apply a vacuum of approximately 0.7 mbar, without drying down the acid.

h) Hydrolyse the sample for 20 hours at 105 °C.

i) After hydrolysis, dry the samples under vacuum in the hydrolysis workstation.

j) Add 500 µL 67% acetonitrile/33% water/0.1% formic acid to each sample.

k) Vortex until sample has detached from the glass (use pipette tip if needed) and transfer suspension to spin filter tube with glass Pasteur pipette.

l) Centrifuge sample for 5 minutes at maximum speed in a micro centrifuge.

m) Transfer filtrate to a glass vial.

n) Add again 500 µL 67% acetonitrile/33% water/0.1% formic acid to each sample tube.

o) Vortex and transfer suspension to same spin filter tube.

p) Centrifuge again for 5 minutes at maximum speed.

q) Transfer filtrate to the same vial, mix and close the vial.

8.2.5.2 Free BMAA and total 0.1 M TCA-soluble BMAA

a) Freeze dry samples to disrupt the cell walls.
b) Weigh 12.5 mg into a plastic 2 ml tube.
c) Add 50 µL of D_3BMAA spike solution
d) Add 600 µL of 0.1 M TCA.
e) Vortex for 1 minute and leave for 10 minutes at room temperature.
f) Vortex again for 1 minute and centrifuge for 5 minutes at maximum speed.
g) Transfer supernatant to a spin filter tube.
h) Centrifuge the spin filter tube for 5 minutes at maximum speed.
i) Transfer filtrate to a new plastic 2 ml tube.
j) Add again 600 µL of 0.1 M TCA to the sample.
k) Vortex for 1 minute and centrifuge for 5 minutes at maximum speed.
l) Transfer the second supernatant to the same spin filter tube.
m) Centrifuge the spin filter tube again for 5 minutes at maximum speed.
n) Transfer the second filtrate to the same tube as the first filtrate (total volume 1250 µL)
o) Vortex and split the extract in two fractions:

Free BMAA:
p) Transfer 500 µL of the extract to a new plastic 2 ml tube.
q) Dry these samples down in speedvac.
r) Add 500 µL of a 67% acetonitrile/33% water/0.1% formic acid solution to each tube.
s) Vortex for 2 minutes and transfer samples to HPLC/UHPLC vials for analysis.

Total soluble BMAA:
t) Transfer 500 µL of the extract to a small glass tube for hydrolysis.
u) Put the samples in the freezer until they are completely frozen and subsequently freeze dry them (overnight).
v) Perform hydrolysis and reconstitute as described for total BMAA (steps **8.2.5.1.e** to **8.2.5.1.q**) but use 2 times 250 µL of 67% acetonitrile/33% water/0.1% formic acid (final volume in HPLC/UHPLC vial is 500 µL).

8.2.5.3 Bound BMAA in the precipitate

Bound BMAA in the pellet created during 0.1 M TCA extraction can best be determined by extracting another 1 mg of lyophilized sample as described in the following steps.

    a) Freeze dry samples to disrupt the cell walls.
    b) Weigh 1 mg into a plastic 2 ml tube.
    c) Add 150 µL of 0.1 M TCA.
    d) Vortex for 1 minute and leave for 10 minutes at room temperature.
    e) Vortex again for 1 minute and centrifuge for 5 minutes at maximum speed.
    f) Discard the supernatant.
    g) Add again 150 µL of 0.1 M TCA to the sample.
    h) Vortex for 1 minute and centrifuge for 5 minutes at maximum speed.
    i) Discard the supernatant.
    j) Transfer the pellet to small glass tube for hydrolysis, use 20 mM HCl to make sure all material is transferred.
    k) Add 40 µL D_3BMAA spike.
    l) Dry the sample under vacuum in the hydrolysis workstation.
    m) Perform hydrolysis and reconstitute as described for total BMAA (steps **8.2.5.1.e** to **8.2.5.1.q**).

Alternatively, the pellet created during the extraction for free and total 0.1 M TCA soluble BMAA (**8.2.5.2**) can be transferred to small glass tubes, dried and hydrolysed as described in steps **8.2.5.1.d** to **8.2.5.1.q**. In this case, a portion of the pellet equivalent to 1 mg of dry weight should be used and D_3BMAA should be added to the pellet after it was transferred to the tube for hydrolysis.

8.2.6. Chromatography

Underivatised BMAA can effectively be separated from DAB and AEG by hydrophilic liquid interaction chromatography (HILIC). For HPLC equipment, chromatography can be performed on a 2.1 × 150 mm, 5 µm ZIC-HILIC column (SeQuant, Sweden) at 40 °C. Use an injection volume of 5 µl and a flow rate of 0.4 ml min^{-1}. Mobile phases are acetonitrile with 0.1 % formic acid (eluent A) and water with 0.1% formic acid (eluent B). Use the following elution program [93]:

- 0–2 min: 95% A
- 4 min: 65% A
- 8–17 min 55% A
- 17–23 min 95% A (equilibration)

with linear decreases between the time steps and directing the flow to waste during the first 4 and last 6 minutes. Chromatograms of a calibration standard and a cycad seed sarcotesta

extracted for free BMAA are shown in Figure 8.2. Separation from isobaric compounds other than DAB and AEG is not described in this SOP, but see [104] for more information.

For LC systems operated at higher pressure, similar columns, but with smaller particle diameter can be used, and the elution program should be adjusted accordingly.

Tips for HILIC chromatography:
  i. The solution used for dissolving the samples and calibration standards should have a low water content to ensure good retention.
  ii. Elute under acidic conditions to ensure BMAA is positively charged and will be well retained on the column.
  iii. Using a relatively high percentage of water at the end of each run and a sufficient equilibration time before the next injection ensures retention time stability.

8.2.7 Detection by MS/MS

Settings of the tandem mass spectrometer should be optimized for the specific equipment used, commercially available standards can be used for this. For detection and quantification of BMAA, electrospray ionisation (ESI) in positive mode is usually employed. The transitions that should be monitored in multiple reaction mode are displayed in Table 8.1. Preferably, two qualifiers are used for BMAA identification. For reliable identification, it is recommended to set the resolution of both mass filters as narrow as possible.

Table 8.1. MS/MS transitions for the analysis of underivatised D_3BMAA, BMAA, DAB and AEG.

Compound	Precursor m/z [a]	Quantifier m/z	Qualifier m/z	Qualifier m/z
D_3BMAA	122.1	105.1	88.1	76.2
BMAA	119.1	102.1	88.1	76.2
DAB	119.1	101.1	74.2	-
AEG	119.1	102.1	-	-

[a] mass-to-charge ratio

For BMAA identification, the following criteria should be met for each sample:

  i. BMAA retention time should be similar to the D_3BMAA retention time in the same sample.
  ii. The signal to noise ratio of all product ions should be at least 3.
  iii. The ratio between m/z 102.1 and m/z 88.1 should not deviate more than 20% from the ratio between the same ions in the BMAA calibration standards.
  iv. The ratio between m/z 102.1 and m/z 76.2 should not deviate more than 20% from the ratio between the same ions in the BMAA calibration standards.
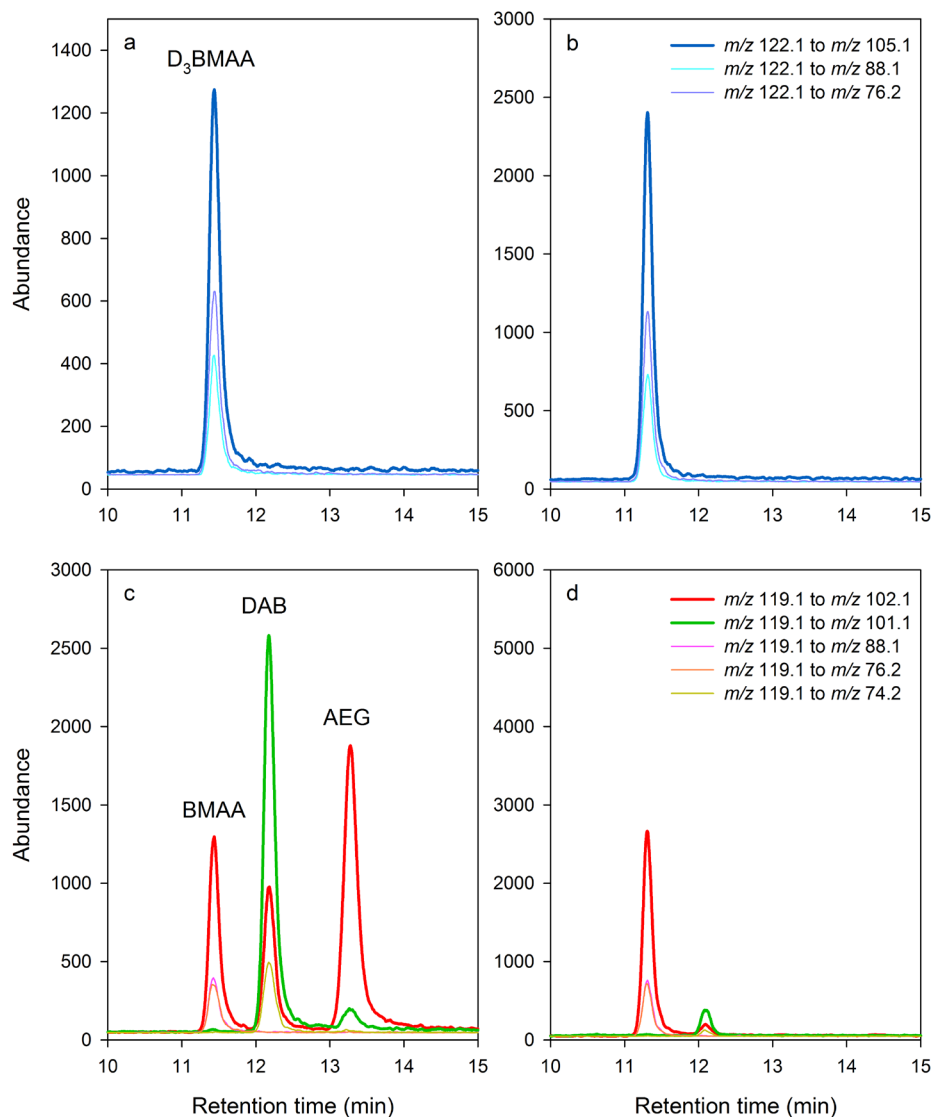
Figure 8.2. Chromatograms of a 50 ng/mL BMAA, D₃BMAA, DAB and AEG standard (panel a and c) and a cycad seed sarcotesta (_Cycas micronesica_), extracted for free BMAA as positive control (8.1 mg used, concentration determined at 11.4 µg/g) and spiked with D₃BMAA at 80 ng/mL (panel b and d). Quantifiers are indicated by bold lines, qualifiers are indicated by normal lines. Chromatography was performed as described in Section 8.2.6 on an Agilent 1260 LC. Compounds were detected on an Agilent 6460 triple quadrupole mass spectrometer with the following settings: drying gas temperature 230 °C, sheath gas temperature 250 °C, drying gas flow and sheath gas flow 12 l/min, nebulizer pressure 40 psi, capillary voltage 2500 V, nozzle voltage 500 V. Both quadrupoles were operated in unit mode. Fragmentor voltage was 50 V, collision energy was 4 V for the transitions m/z 122.1 to 105.1 and m/z 119.1 to 102.1, 101.1 and 74.2 and was 8 V for all other transitions.

8.3 QUANTIFICATION

For BMAA quantification, a calibration curve containing BMAA and D_3BMAA should be constructed. The solvent should be the same as the one used for the samples (e.g. a 67% acetonitrile/33% water/0.1% formic acid solution). Also add DAB and AEG standards to the calibration curve, either for semi-quantification of these compounds, or to make sure that no co-elution with BMAA has taken place. As an example, a dilution series of 0, 0.5, 1, 5, 10, 50 and 100 µg/L could be used, in which each standard contains the reported concentration of each analysed compound (BMAA, D_3BMAA, DAB and AEG).

The LC-MS/MS worklist should start and end with calibration standards, as well as after a fixed number of injected samples (e.g. 10), a set of calibration standards should be analysed In this way, it is possible to correct for changes in signal intensity that might occur during the run. Include vials with only solvent to check for signal carry over.

Quantify BMAA by determining the BMAA and D_3BMAA concentration of each sample against the external calibration curves (signal to noise ratio should be at least 10 for the quantifier ions). Next, calculate D_3BMAA recovery for each sample, and correct the determined BMAA concentration for this value. Finally, correct for the exact amount of sample used, the final volume in which the extract was dissolved before analysis, and in case of free and total 0.1 M TCA-soluble BMAA, the fraction of the extract used; 500/1250 µl/µl (steps **8.2.5.2.p** and **8.2.5.2.t)** (Equation 8.1).
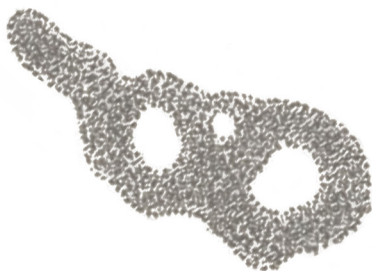
$$[BMAA]_{sample} = [BMAA]_{analysed} \times \frac{[D_3BMAA]_{expected}}{[D_3BMAA]_{analysed}} \times \frac{V_{in\,vial}}{W_{sample}} \times \frac{V_{total\,extract}}{V_{used\,extract}} \quad \text{Equation 8.1}$$

In which $[BMAA]_{sample}$ is expressed as µg/g, $[BMAA]_{analysed}$, $[D_3BMAA]_{expected}$ and $[D_3BMAA]_{analysed}$ as ng/mL, $V_{in\,vial}$ as mL, W_{sample} as mg and $V_{total\,extract}$ and $V_{used\,extract}$ as µL.
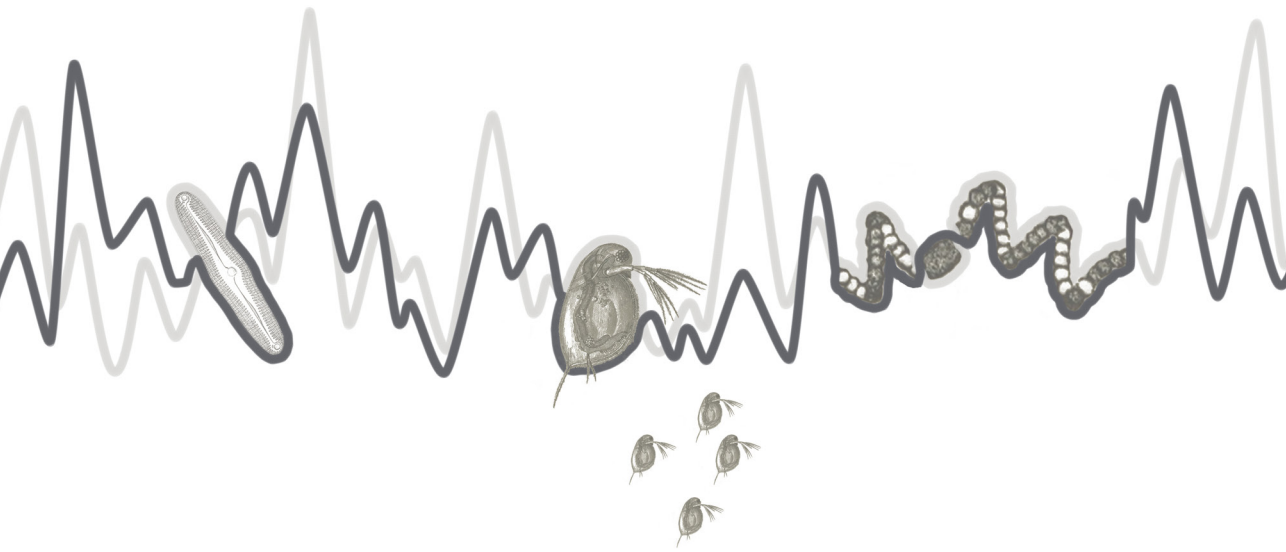
"…. that I do not think that I know what I do not know."

Plato, Apology

CHAPTER 9

S YNTHESIS

Below, the main findings of this thesis will be integrated and discussed.

9.1 BMAA ANALYSIS

Most of the discrepancy in reported BMAA concentrations is caused by the different ways in which BMAA analysis is performed (Chapters 3 and 5). Generally, highest BMAA concentrations are found by methods using optical detection for quantification (Chapter 5). As shown in this thesis, these methods lack selectivity for BMAA, and risk misidentification and overestimation of BMAA concentrations (Chapter 3). It is therefore recommended to use more selective techniques like tandem mass spectrometry for BMAA quantification (Chapter 3, [134]), and at present, LC-MS/MS is the most applied technique (Chapter 5). A diversity of derivatisation techniques can be used in combination with LC-MS/MS analysis of BMAA (e.g. Chapter 3, [100, 145]) and the performance of underivatised LC-MS/MS methods can be good as well (e.g. Chapters 3 and 7, [103, 199]). If each method is properly applied, there seems to be no scientific support for the suggestion that AQC derivatised LC-MS/MS analysis would lead to false positives [77], but neither for the claims that underivatised LC-MS/MS methods will greatly underestimate BMAA concentrations [88] or that these methods simply are inferior to AQC derivatised LC-MS/MS methods [84]. LC-MS/MS methods are still improving: isotope dilution is more and more used for quantification (e.g. [76, 93, 103, 135]), and is now easier to perform as D_3BMAA has become commercially available. In addition, possible interferences of structural isomers are investigated [97, 104], and new methods of separation are explored [104, 202], as are alternative derivatisation techniques [203].

During sample workup, it is important that the different forms in which BMAA can be present are considered (see Figure 1.4). The recent attention for soluble bound BMAA in cycad seeds (Chapter 7 and [92]), periphyton [198], plankton [98, 198], and bivalves [89, 103, 198] calls for revision of the relatively common extraction strategy of determining free BMAA in the aqueous extract and precipitated bound ("protein associated") BMAA in the hydrolysed pellet (e.g. Chapter 2 and [33, 35, 187, 189, 190, 204]). When only free and precipitated bound BMAA are determined, the soluble bound fraction will be overlooked. As long as the structure(s) of soluble bound BMAA are unknown and its extraction is not yet optimised, this problem can be overcome by either hydrolysing the aqueous extract to include total soluble BMAA as an isolated fraction, or to hydrolyse all of the sample for total BMAA analysis (Chapter 9). In addition, it should be checked whether "free" BMAA is not (partly) formed during 0.1 M TCA extraction, as extraction with another dilute acid (20 mM HCl at 5 °C) resulted in a slow release of free BMAA from soluble bound forms in mussels [89]. Similarly, more free BMAA was found in 0.1 M TCA *Skeletonema marinoi* extracts incubated for 48 hours at 4 °C than in similar extracts incubated for 24 hours under the same conditions [200], which might be attributed to the same process. The robustness of 6 M HCl hydrolysis should also be investigated, as within-laboratory reproducibility of total BMAA in

cycad seed sarcotestas was much lower than within-laboratory reproducibility of free BMAA in the same samples (Chapter 7). This finding points at a limitation of using isotope dilution for quantification of total BMAA, as the addition of free D_3BMAA before hydrolysis did not seem to accurately correct for the release of BMAA from bound forms (Chapter 7).

No standardized method for BMAA analysis is at present available, and inter-laboratory comparison studies are required to compare method performance (Chapter 5). The availability of certified reference materials would facilitate such studies, but no reference material has been developed for BMAA yet.

While LC-MS/MS based methods are recommended for their sensitivity and selectivity, these techniques require expensive equipment, specialised operators and in some cases a rather extensive sample preparation. A faster and cheaper method could therefore be useful for screening purposes, so that the number of samples for LC-MS/MS analysis can be reduced. In microcystin research, ELISA kits are frequently used for fast and sensitive analyses (e.g., [205-207]) and in 2012, an ELISA for BMAA detection became commercially available. However, this assay showed high cross reactivity in a diversity of sample types, and should be further developed before application (Chapter 4).

9.2 REPORTING

In addition to the use of inappropriate analytical techniques, poor reporting of analytical results and selective discussion of scientific literature has further created confusion in BMAA research (Chapter 5). One major problem is that for many studies, the identification of BMAA by selective analytical methods cannot be verified because chromatograms are not presented, are incomplete or contain errors. In addition, some studies create a biased view by only citing studies in which BMAA was detected. Finally, the interpretation of BMAA literature was hindered by unclear or unsupported conclusions in some key articles and lack of a critical evaluation of the presented work (Chapter 5). To progress BMAA research in aquatic systems, it is therefore crucial that the use of appropriate methods is combined with correct and complete description of research. This includes an extensive and correct description of methods and results, with special attention to recovery data, quantification procedure and identification. Furthermore, the work should be critically evaluated and should be put it in its scientific context. The validity of published studies lacking information on BMAA identification could be checked if the analytical results would be disclosed, e.g. by deposition in openly available data repositories. Alternatively, comparative analyses could be performed if the sample material used in these studies would be made available.

9.3 PRESENCE OF BMAA IN AQUATIC SYSTEMS

What is really known about the presence of BMAA in aquatic ecosystems, based on studies that used appropriate analytical techniques and that correctly presented their work, is that

BMAA can indeed be present in phytoplankton and higher organisms, likely at µg/g DW concentrations or lower (Chapter 5). More work still needs to be done to identify the BMAA producers in aquatic systems. The detection of BMAA in axenic phytoplankton cultures would give a good indication of whether BMAA is indeed produced by phytoplankton or by their associated bacteria. So far, BMAA has been found in axenic cultures of cyanobacteria [94] and diatoms [98], but independent confirmation of these findings is required. Dinoflagellates have also been suggested to produce BMAA [208], but this hypothesis needs to be tested on axenic cultures. Identification of the genes responsible for BMAA synthesis would greatly assist in assigning BMAA production to specific species or strains, but work in this area is still premature.

Interestingly, BMAA has only once been found in cyanobacteria or cyanobacteria dominated samples analysed by underivatised LC-MS/MS (Chapter 2), while it has more frequently been found in cyanobacteria dominated samples analysed by derivatised LC-MS/MS (e.g. [83, 94, 101, 135, 164]). As shown in Chapter 2, free BMAA was detected in nine out of 21 cyanobacterial field samples from Dutch urban waters, and the highest concentration found was 42 µg/g DW. When an additional set of 52 similar samples was recently analysed for total BMAA following the protocol described in Chapter 7, no BMAA was detected in 48 samples (Table 9.1), and putative BMAA peaks were observed in 4 samples (LOD total BMAA approximately 2.5 µg/g DW). Subsequent analysis of these four samples for free, total soluble and total BMAA (extracted as described in Chapter 9, and analysed as described in Chapter 7), resulted in BMAA detection below quantifiable levels in the only benthic sample, which was dominated by cyanobacteria and diatoms (Figure 9.1), and quantification of 0.6 µg/g DW of total soluble BMAA in a cyanobacteria dominated pelagic sample (Figure 9.1, Table 9.2). These findings are quite in agreement with other studies that did not detect BMAA in cyanobacterial samples by underivatised LC-MS/MS analysis (e.g. [76, 77, 79, 103, 198]) and also with the detection of BMAA in diatoms by underivatised LC-MS/MS analysis (e.g. [198]). However, the number of positive samples and the BMAA concentration found in this recent study are lower than reported in Chapter 2 and it is unclear why the result of these two studies differ this much.
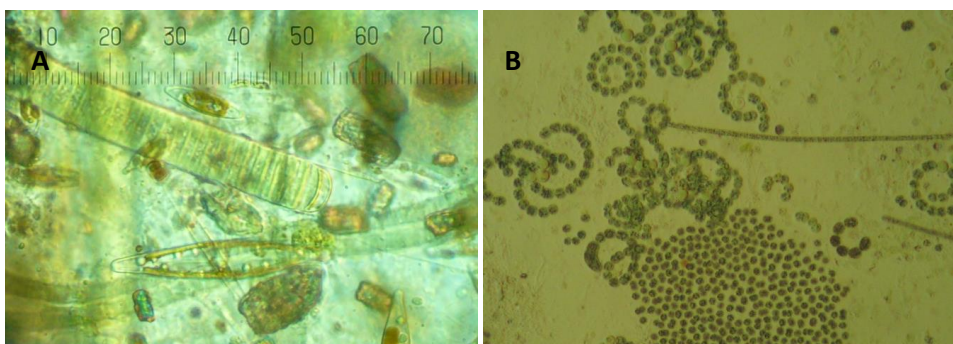
Figure 9.1. Light microscopy images of BMAA containing samples 'Stroombroek' (A) and 'Leusden' (B, Table 9.2). Credit: Miquel Lürling.

Table 9.1. Properties of 47 scum samples and one *Azolla* sample, analysed for total BMAA as described in Chapter 7, in which no BMAA was detected. Samples were lyophilised after collection and were stored at-20 °C before extraction. n.d. means not determined.

Location	Sampling date	Dominant species[1]	Location	Sampling date	Dominant species[1]
Boxtel	25-07-2013	Ma, Mw	Eindhoven	22-08-2013	Df, Dp, Ma
Ede	13-08-2012	PLa	De Meern	17-08-2009	Ma, Dp, Msp
Someren	14-08-2012	Wn, Df	Almere	24-08-2009	Ma, Msp, AZf, Dc
Ursem	07-09-2009	Ma	Tilburg	28-08-2013	Ma, Msp, Wn
Ede	23-08-2013	Df	Beek en Donk	16-07-2010	PLa, Dl, Ma
Breda	21-07-2010	AZf	Barendrecht	09-09-2009	Ma, Mf, AZf
Tilburg	06-08-2009	Ds, Dc	Kralingen	25-10-2012	Msp, Dsp
Tilburg	27-08-2013	n.d.	Middelrode	19-08-2013	Ma, Wn, Mw
Someren	16-07-2010	Ma, Mf	Budel	15-08-2008	Ds, Dc, PLa, AZsp
Huizen	03-07-2009	n.d.	Boxtel	11-09-2010	Wn, Mw, Ma
Tilburg	22-07-2009	n.d.	Beek en Donk	02-08-2011	AZsp, Ma, Dsp
Breda	27-08-2013	n.d.	Wageningen	20-08-2009	Dsp
Someren	16-07-2010	Ma	St. Oedenrode	03-06-2011	Wn, Ma, Mf
Enclave	27-08-2013	n.d.	Prinsenbeek	21-04-2009	AZf
Voordorp	31-08-2009	Msp, Wn	Valkenswaard	02-08-2011	Df
Borculo	15-02-2008	Pr	St. Oedenrode	30-09-2011	Mf, Ma, Wn, Dp
Son	02-08-2011	Dp	St. Oedenrode	15-07-2010	Df, Dp, *Euglena*
Lelystad	24-08-2009	Ma	Bergen op Zoom	06-08-2009	Dc, Ma, Mf, Wn
Nijkerk	23-09-2009	Ma, Df	Budel	22-08-2013	Dc, Ds, Df, Ma, Mf, AZsp
Houten	09-09-2009	Dsp	Bergen op Zoom	27-08-2013	n.d.
Kardinge	15-09-2009	Msp, Dl	St. Oedenrode	05-08-2008	Dc, AZg, Ma, Ssp, ATsp, Lsp, Csp
Wageningen	19-11-2013	*Azolla*	Rotterdam	09-09-2009	Ma, AZf, Dsp
Wageningen	22-07-2010	Ma	Valkenswaard	22-08-2013	Ds
Amsterdam	31-08-2009	Ma	Amersfoort	24-08-2009	AZf, Mb, Ma, Mw, Dp, Df

[1] Species abbreviations: ATsp: *Aphanothece* sp.; AZf: *Aphanizomenon flos-aquae*; AZg: *Aphanizomenon gracile*; AZsp, *Aphanizomenon* sp.; Csp: *Chroococcus* sp.; Dc: *Dolichospermum circinalis*; Df: *Dolichospermum flos-aquae*; Dl: *Dolichospermum lemmermannii*; Dp: *Dolichospermum planktonicum*; Ds: *Dolichospermum spiroides*; Dsp: *Dolichospermum* sp.; Lsp: *Limnothrix* sp.; Ma: *Microcystis aeruginosa*; Mb: *Microcystis botrys*; Mf: *Microcystis flos-aquae*; Msp: *Microcystis* sp.; Mw: *Microcystis wesenbergii*; Osp: *Oscillatoria* sp.; PHsp: *Phormidium* sp.; PLa: *Planktothrix agardhii*; PLr: *Planktothrix rubescens*; Ssp: *Snowella* sp; Wn: *Woronichinia naegeliana*

Table 9.2. Three cyanobacterial scums and a benthic sample analysed for free, total soluble and total BMAA as described in Chapters 7 and 9. n.d. means below limit of detection (LOD), n.q. means below limit of quantification (LOQ). Species codes are explained below Table 9.1.

Location	Sampling date	Dominant species	BMAA (µg/g DW) Free[1]	T.s.[2]	Total[3]
Nieuwkoop	13-08-2009	Wn, Ma, Dp, Ds	n.d.	n.d.	n.d.
Soest	23-09-2009	Ma	n.d.	n.d.	n.d.
Stroombroek	11-06-2013	PHsp, Osp, Dsp, diatoms[4]	n.d.	n.d.	n.q.
Leusden	10-08-2009	Pa, Dc, Df, Ma	n.d.	0.6	n.q.

[1] LOD 0.2 µg/g DW, LOQ 0.5 µg/g DW; [2] Total soluble, LOD 0.2 µg/g DW, LOQ 0.6 µg/g DW;

[3] LOD 2.5 µg/g DW, LOQ 6.2 µg/g DW; [4] Benthic sample

How BMAA production is influenced by environmental conditions is unknown. The experimental setup of one study on BMAA production by cyanobacteria [99] is flawed (Chapter 5), and in another study, no BMAA was detected in the tested cyanobacterial strains [209]. In field situations, the phytoplankton BMAA content was reported to be negatively correlated with the dissolved inorganic nitrogen concentration, but no evidence of correct BMAA identification is provided for this study [210].

BMAA has been found in different trophic levels in the aquatic food web, ranging from phytoplankton to fish (e.g. [83, 100, 145, 197]). Assuming that BMAA is produced by phytoplankton and/or benthic algae, this means that BMAA can be taken up by higher organisms and that it can be transported through the food web. Indeed, zooplankton, mussels and macrophytes can rapidly take up dissolved BMAA [129, 187, 189, 190]. Moreover, BMAA can be maternally transferred (Chapter 6) and it can stay within the body for a longer period of time, as shown for the zooplankter *D. magna* that still contained detectable amounts of BMAA 20 days after exposure (Chapter 6). In most uptake studies so far, organisms have been exposed to dissolved, free BMAA. As BMAA is an endotoxin, exposure studies with BMAA-containing food (e.g. phytoplankton in zooplankton and bivalve studies, zooplankton in fish studies) are required, to determine whether BMAA is as efficiently taken up from ingested biological matrix as from growth medium. For the cyanobacterial toxin microcystin for instance, ingestion of cyanobacterial cells enhanced toxin uptake in *Daphnia*, because an unknown cell compound disrupted *Daphnia*'s gut epithelium [211]. Another reason to perform exposure studies with BMAA-containing food, is that BMAA can be present in different forms in the food source, and that the bioavailability of these distinct forms may differ. *D. magna* has been exposed to cyanobacteria reportedly containing free and precipitated bound BMAA, but after 2 weeks of exposure, neither free nor precipitated bound BMAA could be detected in the *Daphnia* tissue [187]. In another study, Mediterranean mussels (*Mytilus galloprovincialis*) have been exposed to cyanobacteria which reportedly contained traces of BMAA. In this system, BMAA uptake through ingestion of cyanobacteria was described [212], but for both studies it is unclear whether BMAA identification has been performed correctly.

The fate of BMAA in exposed aquatic organisms has rarely been studied. In a study where bivalves were exposed to dissolved BMAA, most BMAA was present in the organisms in a free form. However, the majority of the isotopically labelled BMAA added to the aquaria could not be retrieved after 48 hours [189]. This could either be attributed to instability of BMAA in the exposure media, as observed in Chapter 6, or to the transformation of free BMAA to forms undetectable by the analytical methods used (which only determined free and precipitated bound BMAA), such as soluble bound BMAA. In an experiment with similar setup, but using the macrophyte *Ceratophyllum demersum* as a model species, free BMAA was the most abundant fraction, and both free and precipitated bound BMAA concentrations in the plant dropped during depuration, while no BMAA could be found in the depuration medium [190]. Also in this study, it would have been interesting to see whether the formation of soluble bound BMAA was responsible for the observed decrease in free and precipitated bound BMAA, but this fraction was not analysed. Finally, in *D. magna* exposed to dissolved BMAA, only free BMAA was found in one study ([187], free and precipitated bound BMAA were analysed), while in Chapter 7, soluble bound BMAA (77% of total BMAA) was more abundant in *D. magna* tissue than free BMAA (23%; free, soluble bound and total BMAA were analysed).

9.4 BMAA TOXICITY TO AQUATIC ORGANISMS

As discussed above, BMAA can be present in aquatic systems at different trophic levels, which implies that different groups of aquatic animals are exposed to BMAA. The effect of BMAA on aquatic organisms was first studied on zebrafish larvae (*Danio rerio*). In the presence of bicarbonate, exposure to 5 µg/l dissolved BMAA caused an increase in pericardial oedema, premature hatching and clonus like convulsions (the latter were also observed without the presence of bicarbonate). Exposure levels of 50 µg/l (either with or without bicarbonate) caused spine deformations [168]. The protist *Nassula sorex* showed increased mortality when exposed to dissolved BMAA for 72 hours (LC_{50} 5000 µg/l), and exposure of the brine shrimp *Artemia salina* resulted in the loss of phototactic response at a concentration of 500 µg/l [167]. Exposure of *Daphnia magna* to dissolved BMAA resulted in reduced mobility (EC_{50} after 48 hours at 40 µg/l), decreased brood viability, smaller adults, lighter offspring and decreased population growth (nominal concentration 100 – 110 µg/l, [129] and Chapter 6). In *D. magna*, two generation BMAA exposure lead to higher brood mortality and lower neonate weight than single generation exposure (Chapter 6, nominal concentration 110 µg/l). Interestingly, cyanobacteria can also be negatively affected by BMAA exposure, exposure to 2400 µg/l reduced nitrogen fixation in *Nostoc* [164], and exposure to 500 µg/l reduced the growth rate of *Synechocystis* [213].

In vitro, BMAA can cause oxidative stress [59]. In tests on aquatic species, antioxidant and biotransformation enzyme activity changed in response to BMAA exposure, however, the effect varied between species, exposure times and exposure conditions. In *D. magna* that

were exposed to 100 µg/l dissolved BMAA for 24 h, the activity of the antioxidant enzymes superoxide dismutase and catalase was reduced, as well as of the biotransformation enzyme glutathione S-transferase [187]. Exposure of the same species to concentrations up to 1000 µg/l however did not change the animal's oxidative status, as indicated by hydrogen peroxide concentrations and the thiobarbituric acid reactive substances assay, which was used to indicate lipid peroxidation [187]. Exposure of the macrophyte *Ceratophyllum demersum* to dissolved BMAA in the concentration range of 0.5 to 1.0 µg/l reduced the activity of antioxidant enzymes and glutathione S-transferase, but in this study, the antioxidant concentration in the plant was increased at an exposure to 50 µg/l BMAA [214]. The macrophytes *Lomariopsis lineata*, *Fontinalis antipyretica*, *Riccia fluitans* and *Taxiphyllum barbieri* all responded to BMAA exposure with changes in their antioxidant and biotransformation enzyme activities, but these effects differed in direction and magnitude between the species and the exposure times (1 to 7d) and concentrations used (10 to 100 µg/l) [215]. Similarly, only two out of four bivalve species exposed to BMAA responded in terms of antioxidant and biotransformation enzyme activity, but again variable results were obtained for different exposure times (24 h to 7 d) and concentrations (10 to 500 µg/l) [216]. The mechanism behind these responses still needs to be clarified, and it is also not yet known to which extent BMAA-induced disturbance of the oxidative status of cells contributes to the above summarized effect on animal and population level.

In all ecotoxicological studies performed so far, the aquatic organisms were exposed to relatively high concentrations of dissolved BMAA, and most studies used relatively short (within one generation) exposure times. These tests are valuable for a first screening of effects, and to investigate toxicity mechanisms. For a better estimation of the environmentally relevant toxicological effects of BMAA exposure however, these tests should be complemented with approaches closer to the environmental conditions experienced by the test organisms, such as a more realistic mode of exposure, exposure to multiple stressors and increased exposure time.

BMAA is an endotoxin, and reports on free BMAA in surface waters are rare [217]. Exposure to BMAA containing food (either naturally contaminated or artificially exposed) may lead to different effects than exposure to dissolved BMAA; for instance, uptake may differ when BMAA is present in food (e.g. [211]) and test organisms may change their feeding behaviour, and therefore regulate the amount of toxin ingested, when exposed to toxin containing food [218]. Also, BMAA can be present in different forms in natural food (see section 9.1) and it is still unknown how these forms are taken up and metabolised.

Moreover, under field conditions, aquatic organisms are simultaneously exposed to multiple stressors. For instance, representatives of BMAA-containing phytoplankton groups (diatoms, cyanobacteria) can produce a wide range of known and unidentified toxic compounds [13,

128]. The combined toxicity of BMAA and other algal compounds should therefore be assessed. Combinations of purified BMAA and other known purified phycotoxins, preferably those that could co-occur in a phytoplankton community, can be used for this. BMAA and domoic acid would be a particularly interesting combination, as both compounds cause excitotoxicity and can be present in diatoms. In the only study so far that tested the combined effect of BMAA and microcystin-LR exposure on a filamentous alga [219], BMAA seemed to be better taken up in the presence of microcystin-LR, but it is unclear whether BMAA identification has been correctly performed. The activity of glutathione S-transferase and catalase was higher in the algae that were simultaneously exposed to both toxins than in the algae that were exposed to each toxin alone [219]. To include the effect of unknown algal components present in food, zooplankton or bivalves could be exposed to BMAA-containing phytoplankton strains, with non BMAA containing strains of the same species serving as a control. These type of experiments have already been performed for other phycotoxins [220, 221], and the challenge in this case would be to find enough closely related strains which differ in their BMAA content.

Finally, chronic exposure tests and the inclusion of more sensitive endpoints such as behavioural changes may provide ecologically relevant information. As shown in Chapter 6, two-generation exposure of *D. magna* to dissolved BMAA had a slightly more negative effect on reproduction than single generation exposure, although this did not result in lower population growth rates.

9.5 OUTLOOK

The main question in BMAA research is to what extent BMAA constitutes a human health risk. To answer this question, the relation between BMAA exposure and the onset of the neurodegenerative diseases ALS, PD and AD should be established. And although much work is performed to answer this question, additional evidence is still needed (Section 1.3). In addition to the studies on the neurotoxicity of BMAA, exposure pathways are quantified. In recent studies, BMAA is consistently found in human food such as fish, mussels and crabs at µg/g (dry or wet weight) levels or lower [83, 145, 197, 198, 222, 223] and the consumption of aquatic organisms therefore seems to be an important human exposure route for BMAA. Whether cycads and aquatic organisms are the only sources of human BMAA exposure is unclear, as the presence of BMAA in (cycad unrelated) terrestrial food and ecosystems has not been studied. As we are still unaware of how widespread the production of BMAA really is, screening of terrestrial food items might be prudent, as its structural isomer DAB has been found in tomato (unpublished data) and broccoli [94].

Under future conditions, such as climate change and increased eutrophication, cyanobacteria are expected to become more dominant in freshwater lakes [224, 225]. Also in marine systems, the phytoplankton composition is expected to change [226], although it is at present

not possible to accurately predict the future situation [227]. How these future changes in phytoplankton biomass and composition affect the occurrence of BMAA in aquatic systems is unknown. The total amount of toxin present in a system depends on the abundance of toxin producing species, and the amount of toxin produced by these species under given environmental conditions. Of these two, the abundance of toxin producing species is the most important factor, as strain specific biomass can vary orders of magnitude within a season. The within strain environmental modulation of BMAA production is still unknown, but based on the variation observed in the production of (other) cyanobacterial toxins [150, 228-231], it is expected to make only a small contribution to the total variation.

In conclusion, many questions regarding human exposure to BMAA and the possible health risks associated with it still require an answer. Given the possible severe implications of BMAA exposure, research on the BMAA-neurodegenerative diseases hypothesis needs to be continued. In future research, care should be taken that sound analytical methods are used, and that the research is correctly reported and critically evaluated. In addition, a more constructive discussion and better cooperation between different researchers is required to progress this field.

Eutrophication is a major water quality issue and in many aquatic systems, it leads to the proliferation of toxic phytoplankton species. The neurotoxin β-*N*-methylamino-L-alanine (BMAA) is one of the compounds that can be present in phytoplankton. BMAA has been suggested to play a role in the neurodegenerative diseases Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis, although this hypothesis still needs to be confirmed. It is expected that the main human exposure pathways to BMAA are through direct contact with BMAA containing phytoplankton and through ingestion of BMAA contaminated food, such as fish and shellfish. However, reports on the occurrence of BMAA in aquatic systems have been conflicting and the cause of these reported differences was heavily debated. The use of different analytical methods seems to play a crucial role in the observed discrepancies, but initially, there was little consensus on which method produced most reliable results. The objectives of the work presented in this thesis therefore were to find out what has caused the differences in published results on BMAA concentrations, and to identify and produce reliable data on the presence of BMAA in aquatic systems. In addition, I aimed to determine the effect of BMAA exposure on a key species in many freshwater ecosystems, the grazer *Daphnia magna*.

The performances of different analytical techniques were compared, and LC-MS/MS analysis, either preceded by derivatisation or not, was found to produce most reliable results. LC-FLD and ELISA should not be used for BMAA analysis, as both methods risk misidentifying BMAA or overestimating its concentrations due to their low selectivity. When reviewing literature on the presence of BMAA in aquatic systems, it was found that the observed discrepancies in results could be explained by the use of unselective analytical methods in some studies, and by severe reporting deficiencies in others. When only studies that used appropriate analytical techniques and that correctly reported their work were taken into account, it was shown that BMAA could be present in phytoplankton and higher aquatic organisms, in concentrations of μg/g dry weight or lower. These results are in agreement with our findings of BMAA in cyanobacterial scums from Dutch urban waters. In a 2008 screening, BMAA was found to be present in 9 out of 21 analysed cyanobacterial scums, at concentrations ranging from 4 to 42 μg/g dry weight. When this screening was repeated 8 years later with 52 similar samples, BMAA was detected below the quantification limit in one sample and quantified in another sample at 0.6 μg/g dry weight.

In order to perform the work presented in this thesis, sensitive and selective analytical methods, mostly based on LC-MS/MS analysis without derivatisation, were developed. This resulted in a standard operating procedure for the underivatised LC-MS/MS analysis of BMAA in cyanobacteria. Also, a CYANOCOST initiated workshop was given, in which a group of scientists from 17 independent laboratories evaluated LC-MS/MS based methods

in different matrices. A bound BMAA from found in the supernatant was the most abundant fraction in the positive samples that were tested: cycad seed, seafood and exposed *D. magna*. In addition, it was found that the deuterated internal standard used for quantification was not a good indicator for the release of BMAA from bound forms, resulting in unprecise quantification of total BMAA.

BMAA was found to reduce survival, somatic growth, reproduction and population growth in *D. magna*. Animals did not adapt to BMAA exposure: exposed animals born from exposed mothers had a lower brood viability and neonate weight than animals exposed to BMAA, but born from unexposed mothers. In addition, *D. magna* was shown to take up BMAA from the growth medium and to transfer it to its offspring. *D. magna* therefore might be an important vector for BMAA transfer along the pelagic food chain, but whether BMAA plays a role in preventing zooplankton from controlling cyanobacterial blooms needs further investigation.

Although BMAA research has much progressed between the start of this thesis' work and its completion, some important questions still require an answer. Most urgently, it should be determined whether BMAA is indeed involved in the neurological diseases mentioned above, and if so, which doses trigger the onset of these diseases. Human exposure pathways should then be more systematically quantified, and it might be prudent to investigate if the occurrence of BMAA is restricted to aquatic systems, or whether sources from terrestrial systems contribute to BMAA exposure as well.

1. Harrison, P.A., M. Vandewalle, M.T. Sykes, P.M. Berry, R. Bugter, F. de Bello, C.K. Feld, U. Grandin, R. Harrington, J.R. Haslett, R.H.G. Jongman, G.W. Luck, P.M. da Silva, M. Moora, J. Settele, J.P. Sousa, and M. Zobel, *Identifying and prioritising services in European terrestrial and freshwater ecosystems.* Biodiversity and Conservation, 2010. **19**(10): p. 2791-2821.

2. Barbier, E.B., S.D. Hacker, C. Kennedy, E.W. Koch, A.C. Stier, and B.R. Silliman, *The value of estuarine and coastal ecosystem services.* Ecological Monographs, 2011. **81**(2): p. 169-193.

3. Jackson, R.B., S.R. Carpenter, C.N. Dahm, D.M. McKnight, R.J. Naiman, S.L. Postel, and S.W. Running, *Water in a changing world.* Ecological Applications, 2001. **11**(4): p. 1027-1045.

4. Dudgeon, D., A.H. Arthington, M.O. Gessner, Z.I. Kawabata, D.J. Knowler, C. Lévêque, R.J. Naiman, A.H. Prieur-Richard, D. Soto, M.L.J. Stiassny, and C.A. Sullivan, *Freshwater biodiversity: Importance, threats, status and conservation challenges.* Biological Reviews of the Cambridge Philosophical Society, 2006. **81**(2): p. 163-182.

5. Worm, B., E.B. Barbier, N. Beaumont, J.E. Duffy, C. Folke, B.S. Halpern, J.B.C. Jackson, H.K. Lotze, F. Micheli, S.R. Palumbi, E. Sala, K.A. Selkoe, J.J. Stachowicz, and R. Watson, *Impacts of biodiversity loss on ocean ecosystem services.* Science, 2006. **314**(5800): p. 787-790.

6. Anderson, D.M., P.M. Glibert, and J.M. Burkholder, *Harmful algal blooms and eutrophication: Nutrient sources, composition, and consequences.* Estuaries, 2002. **25**(4): p. 704-726.

7. Smith, V.H., *Eutrophication of freshwater and coastal marine ecosystems: A global problem.* Environmental Science and Pollution Research, 2003. **10**(2): p. 126-139.

8. Sommer, U., R. Adrian, L. De Senerpont Domis, J.J. Elser, U. Gaedke, B. Ibelings, E. Jeppesen, M. Lürling, J.C. Molinero, W.M. Mooij, E. Van Donk, and M. Winder, *Beyond the plankton ecology group (PEG) model: Mechanisms driving plankton succession*, in *Annual Review of Ecology, Evolution, and Systematics*. 2012. p. 429-448.

9. Wilson, A.E., O. Sarnelle, and A.R. Tillmanns, *Effects of cyanobacterial toxicity and morphology on the population growth of freshwater zooplankton: Meta-analyses of laboratory experiments.* Limnology and Oceanography, 2006. **51**(4): p. 1915-1924.

10. Ger, K.A., L.A. Hansson, and M. Lürling, *Understanding cyanobacteria-zooplankton interactions in a more eutrophic world.* Freshwater Biology, 2014. **59**(9): p. 1783-1798.

11. Chorus, I. and J. Bartram, eds. *Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management*. 1999, WHO.

12. Scheffer, M., *Ecology of Shallow Lakes*. Population and Community Biology Series, ed. M.B. Usher. 1998, London: Chapman & Hall.

13. Landsberg, J.H., *The effects of harmful algal blooms on aquatic organisms.* Reviews in Fisheries Science, 2002. **10**(2): p. 113-390.

14. Hudnell, H.K., *Cyanobacterial harmful algal blooms, state of the science and research needs*. Advances in experimental medicine and biology. Vol. 619. 2008, New York: Springer.

15. MacKintosh, C., K.A. Beattie, S. Klumpp, P. Cohen, and G.A. Codd, *Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants.* FEBS Letters, 1990. **264**(2): p. 187-192.

16. Holmes, C.F.B., H.A. Luu, F. Carrier, and F.J. Schmitz, *Inhibition of protein phosphatases-1 and -2A with acanthifolicin: Comparison with diarrhetic shellfish toxins and identification of a region on okadaic acid important for phosphatase inhibition.* FEBS Letters, 1990. **270**(1–2): p. 216-218.

17. Terlau, H., S.H. Heinemann, W. Stühmer, M. Pusch, F. Conti, K. Imoto, and S. Numa, *Mapping the site of block by tetrodotoxin and saxitoxin of sodium channel II.* FEBS Letters, 1991. **293**(1–2): p. 93-96.

18. Mahmood, N.A. and W.W. Carmichael, *The pharmacology of anatoxin-a(s), a neurotoxin produced by the freshwater cyanobacterium Anabaena flos-aquae NRC 525-17.* Toxicon, 1986. **24**(5): p. 425-434.

19. Jeffery, B., T. Barlow, K. Moizer, S. Paul, and C. Boyle, *Amnesic shellfish poison.* Food and Chemical Toxicology, 2004. **42**(4): p. 545-557.

20. Zurawell, R.W., H. Chen, J.M. Burke, and E.E. Prepas, *Hepatotoxic cyanobacteria: A review of the biological importance of microcystins in freshwater environments.* Journal of Toxicology and Environmental Health - Part B: Critical Reviews, 2005. **8**(1): p. 1-37.

21. Carmichael, W.W., *Health effects of toxin-producing cyanobacteria: "The CyanoHABs".* Human and Ecological Risk Assessment (HERA), 2001. **7**(5): p. 1393-1407.

22. Azevedo, S.M.F.O., W.W. Carmichael, E.M. Jochimsen, K.L. Rinehart, S. Lau, G.R. Shaw, and G.K. Eaglesham, *Human intoxication by microcystins during renal dialysis treatment in Caruaru - Brazil.* Toxicology, 2002. **181-182**: p. 441-446.

23. Bienfang, P.K., S.V. DeFelice, E.A. Laws, B.L. E., R.R. Bidigare, S. Christensen, H. Trapido-Rosenthal, T.K. Hemscheidt, D.J. McGillicuddy Jr., D.M. Anderson, H.M. Solo-Gabriele, A.B. Boehm, and L.C. Backer, *Prominent Human Health Impacts from Several Marine Microbes: History, Ecology, and Public Health Implications.* International Journal of Microbiology, 2011. **2011**: p. 1-15.

24. Ibelings, B.W. and I. Chorus, *Accumulation of cyanobacterial toxins in freshwater "seafood" and its consequences for public health: A review.* Environmental Pollution, 2007. **150**(1): p. 177-192.

25. Van Dolah, F.M. and J.S. Ramsdell, *Review and assessment of in vitro detection methods for algal toxins.* Journal of AOAC International, 2001. **84**(5): p. 1617-1625.

26. Krienitz, L., A. Ballot, K. Kotut, C. Wiegand, S. Pütz, J.S. Metcalf, G.A. Codd, and S. Pflugmacher, *Contribution of hot spring cyanobacteria to the mysterious deaths of Lesser Flamingos at Lake Bogoria, Kenya.* FEMS Microbiology Ecology, 2003. **43**(2): p. 141-148.

27. Landsberg, J.H., L.J. Flewelling, and J. Naar, *Karenia brevis red tides, brevetoxins in the food web, and impacts on natural resources: Decadal advancements.* Harmful Algae, 2009. **8**(4): p. 598-607.

28. Faassen, E.J., L. Harkema, L. Begeman, and M. Lurling, *First report of (homo)anatoxin-a and dog neurotoxicosis after ingestion of benthic cyanobacteria in The Netherlands.* Toxicon, 2012. **60**(3): p. 378-384.

29. Lürling, M. and E.J. Faassen, *Dog poisonings associated with a Microcystis aeruginosa bloom in the Netherlands.* Toxins, 2013. **5**(3): p. 556-567.

30. Hoagland, P. and S. Scatasta, *The economic effect of harmful algal blooms.*, in *Ecology of Harmful Algae*, E. Graneli and T. Turner, Editors. 2006, Springer: Berlin. p. 391-402.

31. Vega, A. and E.A. Bell, *α-amino-β-methylaminopropionic acid, a new amino acid from seeds of Cycas circinalis.* Phytochemistry, 1967. **6**: p. 759-762.

32. Cox, P.A., S.A. Banack, and S.J. Murch, *Biomagnification of cyanobacterial neurotoxins and neurodegenerative disease among the Chamorro people of Guam.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(23): p. 13380-13383.

33. Cox, P.A., S.A. Banack, S.J. Murch, U. Rasmussen, G. Tien, R.R. Bidigare, J.S. Metcalf, L.F. Morrison, G.A. Codd, and B. Bergman, *Diverse taxa of cyanobacteria produce β-N-methylamino-L-alanine, a neurotoxic amino acid.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(14): p. 5074-5078.

34. Banack, S.A., H.E. Johnson, R. Cheng, and P.A. Cox, *Production of the neurotoxin BMAA by a marine cyanobacterium.* Marine Drugs, 2007. **5**(4): p. 180-196.

35. Metcalf, J.S., S.A. Banack, J. Lindsay, L.F. Morrison, P.A. Cox, and G.A. Codd, *Co-occurrence of β-N-methylamino-L-alanine, a neurotoxic amino acid with other cyanobacterial toxins in British waterbodies, 1990-2004.* Environmental Microbiology, 2008. **10**(3): p. 702-708.

36. Esterhuizen, M. and T.G. Downing, *β-N-methylamino-L-alanine (BMAA) in novel South African cyanobacterial isolates.* Ecotoxicology and Environmental Safety, 2008. **71**(2): p. 309-313.

37. Bradley, W.G. and D.C. Mash, *Beyond Guam: The cyanobacteria/BMAA hypothesis of the cause of ALS and other neurodegenerative diseases.* Amyotrophic Lateral Sclerosis, 2009. **10**(SUPPL. 2): p. 7-20.

38. Attanasio, R. *BMAA, Neurodegenerative Damage, and the Need for Monitoring*. 2014 [cited 2016 20-01-2016]; Available from: http://ieamblog.com/2014/05/16/bmaa-neurodegenerative-damage-and-the-need-for-monitoring/.

39. Ince, P.G. and G.A. Codd, *Return of the cycad hypothesis - Does the amyotrophic lateral sclerosis/parkinsonism dementia complex (ALS/PDC) of Guam have new implications for global health?* Neuropathology and Applied Neurobiology, 2005. **31**(4): p. 345-353.

40. Kurland, L.T. and D.W. Mulder, *Epidemiologic Investigation of Amyotrophic Lateral Sclerosis.* Neurology, 1954. **4**: p. 355-378 and 438-448.

41. Reed, D., D. Labarthe, C. Kuang Ming, and R. Stallones, *A cohort study of amyotrophic lateral sclerosis and parkinsonism-dementia on Guam and Rota.* American Journal of Epidemiology, 1987. **125**(1): p. 92-100.

42. Banack, S.A. and P.A. Cox, *Distribution of the neurotoxic nonprotein amino acid BMAA in Cycas micronesica.* Botanical Journal of the Linnean Society, 2003. **143**(2): p. 165-168.

43. Banack, S.A. and P.A. Cox, *Biomagnification of cycad neurotoxins in flying foxes: Implications for ALS-PDC in Guam.* Neurology, 2003. **61**(3): p. 387-389.

44. Spencer, P.S., P.B. Nunn, J. Hugon, A. Ludolph, and D.N. Roy, *Motorneurone disease on Guam: Possible role of a food neurotoxin.* Lancet, 1986. **327**(8487): p. 965-965.

45. Kisby, G.E. and P.S. Spencer, *Is Neurodegenerative Disease a Long-Latency Response to Early-Life Genotoxin Exposure?* International Journal of Environmental Research and Public Health, 2011. **8**(10): p. 3889-3921.

46. Spencer, P.S., P.B. Nunn, J. Hugon, A.C. Ludolph, S.M. Ross, D.N. Roy, and R.C. Robertson, *Guam amyotrophic lateral sclerosis-Parkinsonism-dementia linked to a plant excitant neurotoxin.* Science, 1987. **237**(4814): p. 517-522.

47. Spencer, P.S., M. Ohta, and V.S. Palmer, *Cycad use and motor neurone disease in Kii Peninsula of Japan.* Lancet, 1987. **330**(8573): p. 1462-1463.

48. Spencer, P.S., V.S. Palmer, A. Herman, and A. Asmedi H, *Cycad use and motor neurone disease in Irian Jaya.* Lancet, 1987. **330**(8570): p. 1273-1274.

49. Wallace, D.C., *A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: A dawn for evolutionary medicine*, in *Annual Review of Genetics*. 2005. p. 359-407.

50. Blennow, K., M.J. de Leon, and H. Zetterberg, *Alzheimer's disease.* Lancet, 2006. **368**(9533): p. 387-403.

51. Whitehouse, P.J., D.L. Price, R.G. Struble, A.W. Clark, J.T. Coyle, and M.R. DeLong, *Alzheimer's disease and senile dementia: Loss of neurons in the basal forebrain.* Science, 1982. **215**(4537): p. 1237-1239.

52. Dauer, W. and S. Przedborski, *Parkinson's disease: Mechanisms and models.* Neuron, 2003. **39**(6): p. 889-909.

53. Rowland, L.P. and N.A. Shneider, *Amyotrophic lateral sclerosis.* New England Journal of Medicine, 2001. **344**(22): p. 1688-1700.

54. Bruijn, L.I., T.M. Miller, and D.W. Cleveland, *Unraveling the mechanisms involved in motor neuron degeneration in ALS*, in *Annual Review of Neuroscience*. 2004. p. 723-749.

55. Martin, L.J., N.A. Al-Abdulla, A.M. Brambrink, J.R. Kiesch, F.E. Sieber, and C. Portera-Cailliau, *Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: A*

perspective on the contributions of apoptosis and necrosis. Brain Research Bulletin, 1998. **46**(4): p. 281-309.

56. Emerit, J., M. Edeas, and F. Bricaire, *Neurodegenerative diseases and oxidative stress.* Biomedicine & Pharmacotherapy, 2004. **58**(1): p. 39-46.

57. Ross, C.A. and M.A. Poirier, *Protein aggregation and neurodegenerative disease.* Nature Medicine, 2004. **10**(SUPPL.): p. S10-S17.

58. Faassen, E.J., *Presence of the Neurotoxin BMAA in Aquatic Ecosystems: What Do We Really Know?* Toxins, 2014. **6**(3): p. 1109-1138.

59. Chiu, A.S., M.M. Gehringer, J.H. Welch, and B.A. Neilan, *Does α-amino-β-methylaminopropionic acid (BMAA) play a role in neurodegeneration?* International Journal of Environmental Research and Public Health, 2011. **8**(9): p. 3728-3746.

60. Karamyan, V.T. and R.C. Speth, *Animal models of BMAA neurotoxicity: a critical review.* Life Sciences, 2008. **82**(5-6): p. 233-46.

61. Weiss, J.H., C.W. Christine, and D.W. Choi, *Bicarbonate dependence of glutamate receptor activation by β-N-methylamino-L-alanine: Channel recording and study with related compounds.* Neuron, 1989. **3**(3): p. 321-326.

62. Rao, S.D., S.A. Banack, P.A. Cox, and J.H. Weiss, *BMAA selectively injures motor neurons via AMPA/kainate receptor activation.* Experimental Neurology, 2006. **201**(1): p. 244-252.

63. Brownson, D.M., T.J. Mabry, and S.W. Leslie, *The cycad neurotoxic amino acid, β-N-methylamino-L-alanine (BMAA), elevates intracellular calcium levels in dissociated rat brain cells.* Journal of Ethnopharmacology, 2002. **82**(2-3): p. 159-167.

64. Lobner, D., P.M.T. Piana, A.K. Salous, and R.W. Peoples, *β-N-methylamino-L-alanine enhances neurotoxicity through multiple mechanisms.* Neurobiology of Disease, 2007. **25**(2): p. 360-366.

65. Dunlop, R.A., P.A. Cox, S.A. Banack, and K.J. Rodgers, *The Non-Protein Amino Acid BMAA Is Misincorporated into Human Proteins in Place of L-Serine Causing Protein Misfolding and Aggregation.* PLoS ONE, 2013. **8**(9).

66. Banack, S.A., T.A. Caller, and E.W. Stommel, *The cyanobacteria derived toxin beta-N-methylamino-L-alanine and amyotrophic lateral sclerosis.* Toxins, 2010. **2**(12): p. 2837-2850.

67. de Munck, E., E. Muñoz-Sáez, B.G. Miguel, M.T. Solas, A. Martínez, and R.M. Arahuetes, *Morphometric and neurochemical alterations found in L-BMAA treated rats.* Environmental Toxicology and Pharmacology, 2015. **39**(3): p. 1232-1245.

68. Cox, P.A., D.A. Davis, D.C. Mash, J.S. Metcalf, and S.A. Banack, *Dietary exposure to an environmental toxin triggers neurofibrillary tangles and amyloid deposits in the brain.* Proceedings of the Royal Society B: Biological Sciences, 2016. **283**(1823).

69. Pablo, J., S.A. Banack, P.A. Cox, T.E. Johnson, S. Papapetropoulos, W.G. Bradley, A. Buck, and D.C. Mash, *Cyanobacterial neurotoxin BMAA in ALS and Alzheimer's disease.* Acta Neurologica Scandinavica, 2009. **120**(4): p. 216-225.

70. Murch, S.J., P.A. Cox, and S.A. Banack, *A mechanism for slow release of biomagnified cyanobacterial neurotoxins and neurodegenerative disease in Guam.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(33): p. 12228-12231.

71. Murch, S.J., P.A. Cox, S.A. Banack, J.C. Steele, and O.W. Sacks, *Occurrence of β-methylamino-L-alanine (BMAA) in ALS/PDC patients from Guam.* Acta Neurologica Scandinavica, 2004. **110**(4): p. 267-269.

72. Montine, T.J., K. Li, D.P. Perl, and D. Galasko, *Lack of β-methylamino-L-alanine in brain from controls, AD, or Chamorros with PDC.* Neurology, 2005. **65**(5): p. 768-769.

73. Snyder, L.R., R. Cruz-Aguado, M. Sadilek, D. Galasko, C.A. Shaw, and T.J. Montine, *Lack of cerebral BMAA in human cerebral cortex.* Neurology, 2009. **72**(15): p. 1360-1361.

74. Snyder, L.R., J.C. Hoggard, T.J. Montine, and R.E. Synovec, *Development and application of a comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry method for the analysis of L-β-methylamino-alanine in human tissue.* Journal of Chromatography A, 2010. **1217**(27): p. 4639-4647.

75. Berntzon, L., L.O. Ronnevi, B. Bergman, and J. Eriksson, *Detection of BMAA in the human central nervous system.* Neuroscience, 2015. **292**: p. 137-147.

76. Rosén, J. and K.E. Hellenäs, *Determination of the neurotoxin BMAA (β-N-methylamino-L-alanine) in cycad seed and cyanobacteria by LC-MS/MS (liquid chromatography tandem mass spectrometry).* Analyst, 2008. **133**(12): p. 1785-1789.

77. Krüger, T., B. Mönch, S. Oppenhäuser, and B. Luckas, *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, 2010. **55**(2-3): p. 547-557.

78. Kubo, T., N. Kato, K. Hosoya, and K. Kaya, *Effective determination method for a cyanobacterial neurotoxin, β-N-methylamino-L-alanine.* Toxicon, 2008. **51**(7): p. 1264-1268.

79. Li, A., H. Fan, F. Ma, P. McCarron, K. Thomas, X. Tang, and M.A. Quilliam, *Elucidation of matrix effects and performance of solid-phase extraction for LC-MS/MS analysis of β-N-methylamino-L-alanine (BMAA) and 2,4-diaminobutyric acid (DAB) neurotoxins in cyanobacteria.* Analyst, 2012. **137**(5): p. 1210-1219.

80. Marler, T.E., L.R. Snyder, and C.A. Shaw, *Cycas micronesica (Cycadales) plants devoid of endophytic cyanobacteria increase in β-methylamino-L-alanine.* Toxicon, 2010. **56**(4): p. 563-568.

81. Snyder, L.R. and T.E. Marler, *Rethinking cycad metabolite research.* Communicative & Integrative Biology, 2011. **4**(1): p. 86-88.

82. Brand, L.E., J. Pablo, A. Compton, N. Hammerschlag, and D.C. Mash, *Cyanobacterial blooms and the occurrence of the neurotoxin, beta-N-methylamino-L-alanine (BMAA), in South Florida aquatic food webs.* Harmful Algae, 2010. **9**(6): p. 620-635.

83. Jonasson, S., J. Eriksson, L. Berntzon, Z. Spáčil, L.L. Ilag, L.O. Ronnevi, U. Rasmussen, and B. Bergman, *Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(20): p. 9252-9257.

84. Banack, S.A., T.G. Downing, Z. Spáčil, E.L. Purdie, J.S. Metcalf, S. Downing, M. Esterhuizen, G.A. Codd, and P.A. Cox, *Distinguishing the cyanobacterial neurotoxin β-N-methylamino-L-alanine (BMAA) from its structural isomer 2,4-diaminobutyric acid (2,4-DAB).* Toxicon, 2010. **56**(6): p. 868-879.

85. Duncan, M.W., *Good mass spectrometry and its place in good science.* Journal of mass spectrometry, 2012. **47**(6): p. 795-809.

86. Norden, *Analysis, occurrence, and toxicity of β-methylaminoalanine (BMAA), A risk for the consumer?* 2007, Norden.

87. Nunn, P.B., P. O'Brien, L.D. Pettit, and S.I. Pyburn, *Complexes of zinc, copper, and nickel with the nonprotein amino acid L-α-amino-β-methylaminopropionic acid: a naturally occurring neurotoxin.* Journal of Inorganic Biochemistry, 1989. **37**(2): p. 175-183.

88. Glover, W.B., C.M. Liberto, W.S. McNeil, S.A. Banack, P.R. Shipley, and S.J. Murch, *Reactivity of β-methylamino-L-alanine in complex sample matrixes complicating detection and quantification by mass spectrometry.* Analytical Chemistry, 2012. **84**(18): p. 7946-7953.

89. Rosén, J., E. Westerberg, S. Schmiedt, and K.E. Hellenäs, *BMAA detected as neither free nor protein bound amino acid in blue mussels.* Toxicon, 2016. **109**: p. 45-50.

90. Glover, W.B., D.C. Mash, and S.J. Murch, *The natural non-protein amino acid N-β-methylamino-L-alanine (BMAA) is incorporated into protein during synthesis.* Amino Acids, 2014. **46**(11): p. 2553-2559.

91. Van Onselen, R., N.A. Cook, R.R. Phelan, and T.G. Downing, *Bacteria do not incorporate β-N-methylamino-L-alanine into their proteins.* Toxicon, 2015. **102**: p. 55-61.

92. Cheng, R. and S.A. Banack, *Previous studies underestimate BMAA concentrations in cycad flour.* Amyotrophic Lateral Sclerosis, 2009. **10**(SUPPL. 2): p. 41-43.

93. Faassen, E.J., F. Gillissen, and M. Lürling, *A comparative study on three analytical methods for the determination of the neurotoxin BMAA in cyanobacteria.* PLoS ONE, 2012. **7**(5).

94. Spáčil, Z., J. Eriksson, S. Jonasson, U. Rasmussen, L.L. Ilag, and B. Bergman, *Analytical protocol for identification of BMAA and DAB in biological samples.* Analyst, 2010. **135**(1): p. 127-132.

95. Banack, S.A., J.S. Metcalf, Z. Spáčil, T.G. Downing, S. Downing, A. Long, P.B. Nunn, and P.A. Cox, *Distinguishing the cyanobacterial neurotoxin β-N-methylamino-L-alanine (BMAA) from other diamino acids.* Toxicon, 2011. **57**(5): p. 730-738.

96. Cervantes Cianca, R.C., M.S. Baptista, L.P. Da Silva, V.R. Lopes, and V.M. Vasconcelos, *Reversed-phase HPLC/FD method for the quantitative analysis of the neurotoxin BMAA (β-N-methylamino-L-alanine) in cyanobacteria.* Toxicon, 2012. **59**(3): p. 379-384.

97. Jiang, L., B. Aigret, W.M. De Borggraeve, Z. Spacil, and L.L. Ilag, *Selective LC-MS/MS method for the identification of BMAA from its isomers in biological samples.* Analytical and Bioanalytical Chemistry, 2012. **403**(6): p. 1719-1730.

98. Jiang, L., J. Eriksson, S. Lage, S. Jonasson, S. Shams, M. Mehine, L.L. Ilag, and U. Rasmussen, *Diatoms: A Novel Source for the Neurotoxin BMAA in Aquatic Environments.* PLoS ONE, 2014. **9**(1): p. e84578.

99. Downing, S., S.A. Banack, J.S. Metcalf, P.A. Cox, and T.G. Downing, *Nitrogen starvation of cyanobacteria results in the production of β-N-methylamino-L-alanine.* Toxicon, 2011. **58**(2): p. 187-194.

100. Lampinen Salomonsson, M., A. Hansson, and U. Bondesson, *Development and in-house validation of a method for quantification of BMAA in mussels using dansyl chloride derivatisation and ultra performance liquid chromatography tandem mass spectrometry.* Analytical Methods, 2013. **5**(18): p. 4865-4874.

101. Roy-Lachapelle, A., M. Solliec, and S. Sauvé, *Determination of BMAA and three alkaloid cyanotoxins in lake water using dansyl chloride derivatisation and high-resolution mass spectrometry.* Analytical and Bioanalytical Chemistry, 2015. **407**(18): p. 5487-5501.

102. Faassen, E.J., F. Gillissen, H.A.J. Zweers, and M. Lürling, *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.* Amyotrophic Lateral Sclerosis, 2009. **10**(SUPPL. 2): p. 79-84.

103. Réveillon, D., E. Abadie, V. Séchet, L. Brient, V. Savar, M. Bardouil, P. Hess, and Z. Amzil, *Beta-N-methylamino-L-alanine: LC-MS/MS optimization, screening of cyanobacterial strains and occurrence in shellfish from Thau, a French Mediterranean Lagoon.* Marine Drugs, 2014. **12**(11): p. 5441-5467.

104. Beach, D.G., E.S. Kerrin, and M.A. Quilliam, *Selective quantitation of the neurotoxin BMAA by use of hydrophilic-interaction liquid chromatography–differential mobility spectrometry–tandem mass spectrometry (HILIC–DMS–MS/MS).* Analytical and Bioanalytical Chemistry, 2015. **407**: p. 8379-8409.

105. Combes, A., S. El Abdellaoui, C. Sarazin, J. Vial, A. Mejean, O. Ploux, and V. Pichon, *Validation of the analytical procedure for the determination of the neurotoxin β-N-methylamino-L-alanine in complex environmental samples.* Analytica Chimica Acta, 2013. **771**(0): p. 42-49.

106. Kaspar, H., K. Dettmer, W. Gronwald, and P. Oefner, *Advances in amino acid analysis.* Analytical and Bioanalytical Chemistry, 2009. **393**(2): p. 445-452.

107. Johnson, H.E., S.R. King, S.A. Banack, C. Webster, W.J. Callanaupa, and P.A. Cox, *Cyanobacteria (Nostoc commune) used as a dietary item in the Peruvian highlands produce the neurotoxic amino acid BMAA.* Journal of Ethnopharmacology, 2008. **118**(1): p. 159-165.

108. Evans, C.S. and E.A. Bell, *Neuroactive plant amino acids and amines.* Trends in Neurosciences, 1980. **3**(3): p. 70-72.

109. Baratova, L., G. Katrukha, and A. Silaev, *Mechanism of the inactivation of the antibiotic polymyxin M.* Chemistry of Natural Compounds, 1966. **2**(4): p. 224-229.

110. Perkins, H.R. and C.S. Cummins, *Chemical structure of bacterial cell walls: Ornithine and 2,4-diaminobutyric acid as components of the cell walls of plant pathogenic corynebacteria.* Nature, 1964. **201**(4924): p. 1105-1107.

111. Moura, S., M.d.A. Ultramari, D.M.L. de Paula, M. Yonamine, and E. Pinto, *^{1}H NMR determination of β-N-methylamino-L-alanine (L-BMAA) in environmental and biological samples.* Toxicon, 2009. **53**(5): p. 578-583.

112. Dossaji, S.F. and E.A. Bell, *Distribution of α-amino-β-methylaminopropionic acid in Cycas.* Phytochemistry, 1973. **12**(1): p. 143-144.

113. Banack, S.A., S.J. Murch, and P.A. Cox, *Neurotoxic flying foxes as dietary items for the Chamorro people, Marianas Islands.* Journal of Ethnopharmacology, 2006. **106**(1): p. 97-104.

114. Dawson, R.M., *The toxicology of microcystins.* Toxicon, 1998. **36**(7): p. 953-962.

115. Dittmann, E., B.A. Neilan, and T. Börner, *Molecular biology of peptide and polyketide biosynthesis in cyanobacteria.* Applied Microbiology and Biotechnology, 2001. **57**(4): p. 467-473.

116. Mash, D.C., J.P. Pablo, S.A. Banack, P.A. Cox, S. Papapetropoulos, and W. Bradley, *Neurotoxic Non-protein Amino Acid BMAA in Brain from Patients Dying with ALS and Alzheimer's Disease.* Neurology, 2008.

117. Bell, E.A., *The discovery of BMAA, and examples of biomagnification and protein incorporation involving other non-protein amino acids.* Amyotrophic Lateral Sclerosis, 2009. **10**(SUPPL. 2): p. 21-25.

118. Duncan, M.W., J.C. Steele, I.J. Kopin, and S.P. Markey, *2-Amino-3-(methylamino)-propanoic acid (BMAA) in cycad flour: An unlikely cause of amyotrophic lateral sclerosis and parkinsonism-dementia of Guam.* Neurology, 1990. **40**(5): p. 767-772.

119. Cox, P.A. and O.W. Sacks, *Cycad neurotoxins, consumption of flying foxes, and ALS-PDC disease in Guam.* Neurology, 2002. **58**(6): p. 956-959.

120. Li, A., Z. Tian, J. Li, R. Yu, S.A. Banack, and Z. Wang, *Detection of the neurotoxin BMAA within cyanobacteria isolated from freshwater in China.* Toxicon, 2010. **55**(5): p. 947-953.

121. Baptista, M.S., R.C.C. Cianca, V.R. Lopes, C.M.R. Almeida, and V.M. Vasconcelos, *Determination of the non protein amino acid β-N-methylamino-L-alanine in estuarine cyanobacteria by capillary electrophoresis.* Toxicon, 2011. **58**(5): p. 410-414.

122. Esterhuizen-Londt, M., S. Downing, and T.G. Downing, *Improved sensitivity using liquid chromatography mass spectrometry (LC-MS) for detection of propyl chloroformate derivatised β-N-methylamino-L-alanine (BMAA) in cyanobacteria.* Water SA, 2011. **37**(2): p. 133-138.

123. Codd, G.A., S.G. Bell, K. Kaya, C.J. Ward, K.A. Beattie, and J.S. Metcalf, *Cyanobacterial toxins, exposure routes and human health.* European Journal of Phycology, 1999. **34**(4): p. 405-415.

124. Waters, *Waters AccQ-Tag Chemistry Package Instruction Manual*. 1993, Milford, USA: Waters Cooperation.

125. Eriksson, J., S. Jonasson, D. Papaefthimiou, U. Rasmussen, and B. Bergman, *Improving derivatisation efficiency of BMAA utilizing AccQ-Tag® in a complex cyanobacterial matrix.* Amino Acids, 2009. **36**(1): p. 43-48.

126. Cohen, S.A. and D.P. Michaud, *Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography.* Analytical Biochemistry, 1993. **211**(2): p. 279-287.

127. Bell, E.A., *Nonprotein amino acids of plants: Significance in medicine, nutrition, and agriculture.* Journal of Agricultural and Food Chemistry, 2003. **51**(10): p. 2854-2865.

128. Sivonen, K. and G.J. Jones, *Cyanobacterial toxins*, in *Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring and Management*, I. Chorus and J. Bartram, Editors. 1999, E&FN Spon: London. p. 41-111.

129. Lürling, M., E.J. Faassen, and J.S.V. Eenennaam, *Effects of the cyanobacterial neurotoxin β-N-methylamino-L-alanine (BMAA) on the survival, mobility and reproduction of Daphnia magna.* Journal of Plankton Research, 2011. **33**(2): p. 333-342.

130. ICH, *Text on Validation of Analytical Procedures Q2A*. 1994, US Food and Drug Administration.

131. ICH, *Guidance for Industry, Q2B Validation of Analytical Procedures: Methodology*. 1996, US Food and Drug Administration.

132. Lurling, M. and W. Beekman, *Palmelloids formation in Chlamydomonas reinhardtii: Defence against rotifer predators?* Annales de Limnologie, 2006. **42**(2): p. 65-72.

133. Cervantes Cianca, R.C., M.S. Baptista, V.R. Lopes, and V.M. Vasconcelos, *The non-protein amino acid β-N-methylamino-L-alanine in Portuguese cyanobacterial isolates.* Amino Acids, 2012. **42**(6): p. 2473-2479.

134. Cohen, S.A., *Analytical techniques for the detection of α-amino-β-methylaminopropionic acid.* Analyst, 2012. **137**(9): p. 1991-2005.

135. Jiang, L., E. Johnston, K.M. Åberg, U. Nilsson, and L.L. Ilag, *Strategy for quantifying trace levels of BMAA in cyanobacteria by LC/MS/MS.* Analytical and Bioanalytical Chemistry, 2012: p. 1-10.

136. Dai, R., H. Liu, J. Qu, X. Zhao, and Y. Hou, *Effects of amino acids on microcystin production of the Microcystis aeruginosa.* Journal of Hazardous Materials, 2009. **161**(2-3): p. 730-736.

137. Zahradníčková, H., P. Hušek, P. Šimek, P. Hartvich, B. Maršálek, and I. Holoubek, *Determination of D- and L-amino acids produced by cyanobacteria using gas chromatography on Chirasil-Val after derivatisation with pentafluoropropyl chloroformate.* Analytical and Bioanalytical Chemistry, 2007. **388**(8): p. 1815-1822.

138. Whiting, M.G., *Toxicity of cycads.* Economic Botany, 1963. **17**: p. 271-302.

139. Duncan, M.W., I.J. Kopin, R.M. Garruto, L. Lavine, and S.P. Markey, *2-Amino-3 (methylamino)-propionic acid in cycad-derived foods is an unlikely cause of amyotrophic lateral sclerosis/parkinsonism.* Lancet, 1988. **332**(8611): p. 631-632.

140. Wilson, J. and C.A. Shaw, *Commentary on: Return of the cycad hypothesis - does the amyotrophic lateral sclerosis/parkinsonism dementia complex (ALS/PDC) of Guam have new implications for global health?* Neuropathology and applied neurobiology, 2006. **32**(3): p. 341-343.

141. Okle, O., K. Stemmer, U. Deschl, and D.R. Dietrich, *L-BMAA induced ER stress and enhanced caspase 12 cleavage in human neuroblastoma SH-SY5Y cells at low nonexcitotoxic concentrations.* Toxicological Sciences, 2013. **131**(1): p. 217-224.

142. Jiang, L. and L.L. Ilag, *Detection of endogenous BMAA in dinoflagellate (Heterocapsa triquetra) hints at evolutionary conservation and environmental concern.* Pubraw Science, 2014. **1(2): p.1-8.**

143. Metcalf, J.S., S.A. Banack, K. Kotut, L. Krienitz, and G.A. Codd, *Amino acid neurotoxins in feathers of the Lesser Flamingo, Phoeniconaias minor.* Chemosphere, 2013. **90**(2): p. 835-839.

144. Mondo, K., N. Hammerschlag, M. Basile, J. Pablo, S.A. Banack, and D.C. Mash, *Cyanobacterial neurotoxin β-N-methylamino-L-alanine (BMAA) in Shark Fins.* Marine Drugs, 2012. **10**(2): p. 509-520.

145. Christensen, S.J., T.K. Hemscheidt, H. Trapido-Rosenthal, E.A. Laws, and R.R. Bidigare, *Detection and quantification of β-methylamino-L-alanine in aquatic invertebrates.* Limnology and Oceanography: Methods, 2012. **10**: p. 891-898.

146. Field, N.C., J.S. Metcalf, T.A. Caller, S.A. Banack, P.A. Cox, and E.W. Stommel, *Linking β-methylamino-L-alanine exposure to sporadic amyotrophic lateral sclerosis in Annapolis, MD.* Toxicon, 2013. **70**(0): p. 179-183.

147. Niedzwiadek, B., P.M. Scott, and B.P.Y. Lau, *Monitoring of shrimp and farmed fish sold in Canada for cyanobacterial toxins.* Journal of Food Protection, 2012. **75**(1): p. 160-163.

148. Scott, P.M., B. Niedzwiadek, D.F.K. Rawnben, and P.Y. Lau, *Liquid chromatographic determination of the cyanobacterial toxin β-N-methylamino-L-alanine in algae Food supplements, freshwater Fish, and Bottled Water.* Journal of Food Protection, 2009. **72**(8): p. 1769-1773.

149. Jiao, Y., Q. Chen, X. Chen, X. Wang, X. Liao, L. Jiang, J. Wu, and L. Yang, *Occurrence and transfer of a cyanobacterial neurotoxin β-methylamino-L-alanine within the aquatic food webs of Gonghu Bay (Lake Taihu, China) to evaluate the potential human health risk.* Science of the Total Environment, 2014. **468-469**: p. 457-463.

150. Van De Waal, D.B., J.M.H. Verspagen, M. Lürling, E. Van Donk, P.M. Visser, and J. Huisman, *The ecological stoichiometry of toxins produced by harmful cyanobacteria: An experimental test of the carbon-nutrient balance hypothesis.* Ecology Letters, 2009. **12**(12): p. 1326-1335.

151. Faassen, E.J. and M. Lürling, *Occurrence of the microcystins MC-LW and MC-LF in Dutch surface waters and their contribution to total microcystin toxicity.* Marine Drugs, 2013. **11**(7): p. 2643-2654.

152. Messineo, V., S. Bogialli, S. Melchiorre, N. Sechi, A. Lugliè, P. Casiddu, M.A. Mariani, B.M. Padedda, A.D. Corcia, R. Mazza, E. Carloni, and M. Bruno, *Cyanobacterial toxins in Italian freshwaters.* Limnologica, 2009. **39**(2): p. 95-106.

153. Faassen, E.J., W. Beekman, and M. Lürling, *Evaluation of a Commercial Enzyme Linked Immunosorbent Assay (ELISA) for the Determination of the Neurotoxin BMAA in Surface Waters.* PLoS ONE, 2013. **8**(6).

154. Kebarle, P. and L. Tang, *From ions in solution to ions in the gas phase: The mechanism of electrospray mass spectrometry.* Analytical Chemistry, 1993. **65**(22): p. 972A-986A.

155. SANCO, *Method validation and quality control procedures for pesticide residues analysis in food and feed*. 2011.

156. Cox, P.A., R. Richer, J.S. Metcalf, S.A. Banack, G.A. Codd, and W.G. Bradley, *Cyanobacteria and BMAA exposure from desert dust: A possible link to sporadic ALS among Gulf War veterans.* Amyotrophic Lateral Sclerosis, 2009. **10**(SUPPL. 2): p. 109-117.

157. Roney, B.R., L. Renhui, S.A. Banack, S. Murch, R. Honegger, and P.A. Cox, *Consumption of fa cai Nostoc soup: A Potential for BMAA exposure from Nostoc cyanobacteria in China?* Amyotrophic Lateral Sclerosis, 2009. **10**(SUPPL. 2): p. 44-49.

158. Bidigare, R.R., S.J. Christensen, S.B. Wilde, and S.A. Banack, *Cyanobacteria and BMAA: Possible linkage with avian vacuolar myelinopathy (AVM) in the south-eastern United States.* Amyotrophic Lateral Sclerosis, 2009. **10**(SUPPL. 2): p. 71-73.

159. Craighead, D., J.S. Metcalf, S.A. Banack, L. Amgalan, H.V. Reynolds, and M. Batmunkh, *Presence of the neurotoxic amino acids β-N-methylamino-L-alanine (BMAA) and 2,4-diamino-butyric acid (DAB) in shallow springs from the Gobi Desert.* Amyotrophic Lateral Sclerosis, 2009. **10**(SUPPL. 2): p. 96-100.

160. Canizares-Villanueva, R.O., A.R. Dominguez, M.S. Cruz, and E. Rios-Leal, *Chemical composition of cyanobacteria grown in diluted, aerated swine wastewater.* Bioresource Technology, 1995. **51**(2-3): p. 111-116.

161. Muro-Pastor, M.I., J.C. Reyes, and F.J. Florencio, *Cyanobacteria Perceive Nitrogen Status by Sensing Intracellular 2-Oxoglutarate Levels.* Journal of Biological Chemistry, 2001. **276**(41): p. 38320-38328.

162. Esterhuizen-Londt, M. and T.G. Downing, *Solid phase extraction of β-N-methylamino-L-alanine (BMAA) from South African water supplies.* Water SA, 2011. **37**(4): p. 523-528.

163. Caller, T.A., J.W. Doolin, J.F. Haney, A.J. Murby, K.G. West, H.E. Farrar, A. Ball, B.T. Harris, and E.W. Stommel, *A cluster of amyotrophic lateral sclerosis in New Hampshire: A possible role for toxic cyanobacteria blooms.* Amyotrophic Lateral Sclerosis, 2009. **10**(SUPPL. 2): p. 101-108.

164. Berntzon, L., S. Erasmie, N. Celepli, J. Eriksson, U. Rasmussen, and B. Bergman, *BMAA inhibits nitrogen fixation in the cyanobacterium nostoc sp. PCC 7120.* Marine Drugs, 2013. **11**(8): p. 3091-3108.

165. Andersen, R.A., *Algal Culturing Techniques*, 2005: Elsevier Academic Press, Burlington, USA.

166. Rippka, R. and A.N.G. Lester Packer, *Isolation and purification of cyanobacteria*, in *Methods in Enzymology*. 1988, Academic Press. p. 3-27.

167. Purdie, E.L., J.S. Metcalf, S. Kashmiri, and G.A. Codd, *Toxicity of the cyanobacterial neurotoxin β-N-methylamino-L-alanine to three aquatic animal species.* Amyotrophic Lateral Sclerosis, 2009. **10**(SUPPL. 2): p. 67-70.

168. Purdie, E.L., S. Samsudin, F.B. Eddy, and G.A. Codd, *Effects of the cyanobacterial neurotoxin β-N-methylamino-L-alanine on the early-life stage development of zebrafish (Danio rerio).* Aquatic Toxicology, 2009. **95**(4): p. 279-284.

169. Snyder, L.R., R. Cruz-Aguado, M. Sadilek, D. Galasko, C.A. Shaw, and T.J. Montine, *Parkinson–dementia complex and development of a new stable isotope dilution assay for BMAA detection in tissue.* Toxicology and Applied Pharmacology, 2009. **240**(2): p. 180-188.

170. Nozal, M.J., J.L. Bernal, M.L. Toribio, J.C. Diego, and A. Ruiz, *Rapid and sensitive method for determining free amino acids in honey by gas chromatography with flame ionization or mass spectrometric detection.* Journal of Chromatography A, 2004. **1047**(1): p. 137-146.

171. Bruland, K.W., J.R. Donat, and D.A. Hutchins, *Interactive Influences of Bioactive Trace Metals on Biological Production in Oceanic Waters.* Limnology and Oceanography, 1991. **36**(8): p. 1555-1577.

172. Dortch, Q., *The interaction between ammonium and nitrate uptake in phytoplankton.* Marine Ecology Progress Series, 1990. **61**: p. 183-201.

173. Orr, P.T. and G.J. Jones, *Relationship Between Microcystin Production and Cell Division Rates in Nitrogen-Limited Microcystis aeruginosa Cultures.* Limnology and Oceanography, 1998. **43**(7): p. 1604-1614.

174. Al-Sammak, M.A., K.D. Hoagland, D.D. Snow, and D. Cassada, *Methods for simultaneous detection of the cyanotoxins BMAA, DABA, and anatoxin- A in environmental samples.* Toxicon, 2013. **76**: p. 316-325.

175. Spencer, P.S., G.E. Kisby, and A.C. Ludolph, *Slow toxins, biologic markers, and long-latency neurodegenerative disease in the western Pacific region.* Neurology, 1991. **41**(5 Suppl 2): p. 62-66; discussion 66.

176. Karlsson, O., L. Jiang, M. Andersson, L.L. Ilag, and E.B. Brittebo, *Protein association of the neurotoxin and non-protein amino acid BMAA (β-N-methylamino-L-alanine) in the liver and brain following neonatal administration in rats.* Toxicology Letters, 2014. **226**(1): p. 1-5.

177. Karlsson, O., N.G. Lindquist, E.B. Brittebo, and E. Roman, *Selective brain uptake and behavioral effects of the cyanobacterial toxin BMAA (β-N-Methylamino-L-alanine) following neonatal administration to rodents.* Toxicological Sciences, 2009. **109**(2): p. 286-295.

178. Andersson, M., O. Karlsson, U. Bergström, E.B. Brittebo, and I. Brandt, *Maternal Transfer of the Cyanobacterial Neurotoxin β-N-Methylamino-L-Alanine (BMAA) via Milk to Suckling Offspring.* PLoS ONE, 2013. **8**(10).

179. Dao, T.S., L.C. Do-Hong, and C. Wiegand, *Chronic effects of cyanobacterial toxins on Daphnia magna and their offspring.* Toxicon, 2010. **55**(7): p. 1244-1254.

180. Lemaire, V., S. Brusciotti, I. van Gremberghe, W. Vyverman, J. Vanoverbeke, and L. De Meester, *Genotype×genotype interactions between the toxic cyanobacterium Microcystis and its grazer, the waterflea Daphnia.* Evolutionary Applications, 2012. **5**(2): p. 168-182.

181. Gustafsson, S., K. Rengefors, and L.A. Hansson, *Increased consumer fitness following transfer of toxin tolerance to offspring via maternal effects.* Ecology, 2005. **86**(10): p. 2561-2567.

182. Ortiz-Rodríguez, R., T. Son Dao, and C. Wiegand, *Transgenerational effects of microcystin-LR on Daphnia magna.* Journal of Experimental Biology, 2012. **215**(16): p. 2795-2805.

183. Jiang, X., W. Yang, S. Zhao, H. Liang, Y. Zhao, L. Chen, and R. Li, *Maternal effects of inducible tolerance against the toxic cyanobacterium Microcystis aeruginosa in the grazer Daphnia carinata.* Environmental Pollution, 2013. **178**(0): p. 142-146.

184. Tollrian, R., *Neckteeth formation in Daphnia pulex as an example of continuous phenotypic plasticity: Morphological effects of Chaoborus kairomone concentration and their quantification.* Journal of Plankton Research, 1993. **15**(11): p. 1309-1318.

185. Begon, M., C.R. Townsend, and J.L. Harper, *Ecology: from individuals to ecosystems*. 4 ed. 2006, Malden, USA: Blackwell Publishing. 738.

186. Meyer, J.S., C.G. Ingersoll, L.L. McDonald, and M.S. Boyce, *Estimating uncertainty in population growth rates: jackknife vs. bootstrap techniques.* 1986. **67**(5): p. 1156-1166.

187. Esterhuizen-Londt, M., C. Wiegand, and T.G. Downing, *β-N-methylamino-L-alanine (BMAA) uptake by the animal model, Daphnia magna and subsequent oxidative stress.* Toxicon, 2015. **100**: p. 20-26.

188. Agrawal, A.A., C. Laforsch, and R. Tollrian, *Transgenerational induction of defences in animals and plants.* Nature, 1999. **401**(6748): p. 60-63.

189. Downing, S., V. Contardo-Jara, S. Pflugmacher, and T.G. Downing, *The fate of the cyanobacterial toxin β-N-methylamino-L-alanine in freshwater mussels.* Ecotoxicology and Environmental Safety, 2014. **101**(1): p. 51-58.

190. Downing, S., M. Esterhuizen-Londt, and T. Grant Downing, *β-N-methylamino-L-alanine (BMAA) metabolism in the aquatic macrophyte Ceratophyllum demersum.* Ecotoxicology and Environmental Safety, 2015. **120**: p. 88-92.

191. Zenk, M.H. and M. Juenger, *Evolution and current status of the phytochemistry of nitrogenous compounds.* Phytochemistry, 2007. **68**(22-24): p. 2757-2772.

192. Lürling, M. and E. Van Donk, *Life history consequences for Daphnia pulex feeding on nutrient-limited phytoplankton.* Freshwater Biology, 1997. **38**(3): p. 693-709.

193. Carotenuto, Y., T. Wichard, G. Pohnert, and W. Lampert, *Life-history responses of Daphnia pulicaria to diets containing freshwater diatoms: Effects of nutritional quality versus polyunsaturated aldehydes.* Limnology and Oceanography, 2005. **50**(2): p. 449-454.

194. Ianora, A. and A. Miralto, *Toxigenic effects of diatoms on grazers, phytoplankton and other microbes: A review.* Ecotoxicology, 2010. **19**(3): p. 493-511.

195. Faassen, E.J., M. García-Altares, M. Mendes e Mello, and M. Lürling, *Trans generational effects of the neurotoxin BMAA on the aquatic grazer Daphnia magna.* Aquatic Toxicology, 2015. **168**: p. 98-107.

196. Esterhuizen, M., S. Pflugmacher, and T.G. Downing, *β-N-Methylamino-L-alanine (BMAA) uptake by the aquatic macrophyte Ceratophyllum demersum.* Ecotoxicology and Environmental Safety, 2011. **74**(1): p. 74-77.

197. Jiang, L., N. Kiselova, J. Rosén, and L.L. Ilag, *Quantification of neurotoxin BMAA (β-N-methylamino-L-alanine) in seafood from Swedish markets.* Scientific Reports, 2014. **4**: p. 6931.

198. Réveillon, D., E. Abadie, V. Séchet, E. Masseret, P. Hess, and Z. Amzil, *β-N-methylamino-L-alanine (BMAA) and isomers: Distribution in different food web compartments of Thau lagoon, French Mediterranean Sea.* Marine Environmental Research, 2015. **110**: p. 8-18.

199. Combes, A., S. El Abdellaoui, J. Vial, E. Lagrange, and V. Pichon, *Development of an analytical procedure for quantifying the underivatized neurotoxin β-N-methylamino-L-alanine in brain tissues.* Analytical and Bioanalytical Chemistry, 2014. **406**(19): p. 4627-4636.

200. Lage, S., A. Burian, U. Rasmussen, P.R. Costa, H. Annadotter, A. Godhe, and S. Rydberg, *BMAA extraction of cyanobacteria samples: which method to choose?* Environmental Science and Pollution Research, 2015.

201. SANCO, *Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed.* 2013.

202. Svoboda, P., A. Combes, J. Petit, L. Nováková, V. Pichon, and BMAALS group, *Synthesis of a molecularly imprinted sorbent for selective solid-phase extraction of β-N-methylamino-L-alanine.* Talanta, 2015. **144**: p. 1021-1029.

203. Andrýs, R., J. Zurita, N. Zguna, K. Verschueren, W.M. De Borggraeve, and L.L. Ilag, *Improved detection of β-N-methylamino-L-alanine using N-hydroxysuccinimide ester of N-butylnicotinic acid for the localization of BMAA in blue mussels (Mytilus edulis).* Analytical and Bioanalytical Chemistry, 2015. **407**(13): p. 3743-3750.

204. Karlsson, O., L. Jiang, L. Ersson, T. Malmström, L.L. Ilag, and E.B. Brittebo, *Environmental neurotoxin interaction with proteins: Dose-dependent increase of free and protein-associated BMAA (β-N-methylamino-L-alanine) in neonatal rat brain.* Scientific Reports, 2015. **5**: p. 15570.

205. Karlsson, K.M., H. Kankaanpää, M. Huttunen, and J. Meriluoto, *First observation of microcystin-LR in pelagic cyanobacterial blooms in the northern Baltic Sea.* Harmful Algae, 2005. **4**(1): p. 163-166.

206. Freitas de Magalhães, V., R. Moraes Soares, and S.M.F.O. Azevedo, *Microcystin contamination in fish from the Jacarepaguá Lagoon (Rio de Janeiro, Brazil): Ecological implication and human health risk.* Toxicon, 2001. **39**(7): p. 1077-1085.

207. Mohamed, Z.A., W.W. Carmichael, and A.A. Hussein, *Estimation of microcystins in the freshwater fish Oreochromis niloticus in an Egyptian fish farm containing a Microcystis bloom.* Environmental Toxicology, 2003. **18**(2): p. 137-141.

208. Lage, S., P.R. Costa, T. Moita, J. Eriksson, U. Rasmussen, and S.J. Rydberg, *BMAA in shellfish from two Portuguese transitional water bodies suggests the marine dinoflagellate Gymnodinium catenatum as a potential BMAA source.* Aquatic Toxicology, 2014. **152**: p. 131-138.

209. Fan, H., J. Qiu, L. Fan, and A. Li, *Effects of growth conditions on the production of neurotoxin 2,4-diaminobutyric acid (DAB) in Microcystis aeruginosa and its universal presence in diverse cyanobacteria isolated from freshwater in China.* Environmental Science and Pollution Research, 2015. **22**(8): p. 5943-5951.

210. Scott, L.L., S. Downing, R.R. Phelan, and T.G. Downing, *Environmental modulation of microcystin and β-N-methylamino-L-alanine as a function of nitrogen availability.* Toxicon, 2014. **87**: p. 1-5.

211. Rohrlack, T., K. Christoffersen, E. Dittmann, I. Nogueira, V. Vasconcelos, and T. Börner, *Ingestion of microcystins by Daphnia: Intestinal uptake and toxic effects.* Limnology and Oceanography, 2005. **50**(2): p. 440-448.

212. Baptista, M.S., R.G.W. Vasconcelos, P.C. Ferreira, C.M.R. Almeida, and V.M. Vasconcelos, *Assessment of the non-protein amino acid BMAA in Mediterranean mussel Mytilus galloprovincialis after feeding with estuarine cyanobacteria.* Environmental Science and Pollution Research, 2015. **22**(16): p. 12501-12510.

213. Downing, S., M. van de Venter, and T.G. Downing, *The Effect of Exogenous β-N-Methylamino-L-alanine on the Growth of Synechocystis PCC6803.* Microbial Ecology, 2012. **63**(1): p. 149-156.

214. Esterhuizen-Londt, M., S. Pflugmacher, and T.G. Downing, *The effect of β-N-methylamino-L-alanine (BMAA) on oxidative stress response enzymes of the macrophyte Ceratophyllum demersum.* Toxicon, 2011. **57**(5): p. 803-810.

215. Contardo-Jara, V., M. Sebastian Funke, A. Peuthert, and S. Pflugmacher, *β-N-Methylamino-L-alanine exposure alters defense against oxidative stress in aquatic plants Lomariopsis lineata, Fontinalis antipyretica, Riccia fluitans and Taxiphyllum barbieri.* Ecotoxicology and Environmental Safety, 2013. **88**: p. 72-78.

216. Contardo-Jara, V., S.K.B. Otterstein, S. Downing, T.G. Downing, and S. Pflugmacher, *Response of antioxidant and biotransformation systems of selected freshwater mussels (Dreissena polymorpha, Anadonta cygnea, Unio tumidus, and Corbicula javanicus) to the cyanobacterial neurotoxin β-N-methylamino-L-alanine.* Toxicological and Environmental Chemistry, 2014. **96**(3): p. 451-465.

217. Al-Sammak, M.A., K.D. Hoagland, D. Cassada, and D.D. Snow, *Co-occurrence of the cyanotoxins BMAA, DABA and anatoxin-a in Nebraska reservoirs, fish, and aquatic plants.* Toxins, 2014. **6**(2): p. 488-508.

218. Demott, W.R., Z. Qing-Xue, and W.W. Carmichael, *Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of Daphnia.* Limnology & Oceanography, 1991. **36**(7): p. 1346-1357.

219. Contardo-Jara, V., S. Kuehn, and S. Pflugmacher, *Single and combined exposure to MC-LR and BMAA confirm suitability of Aegagropila linnaei for use in green liver systems®-A case study with cyanobacterial toxins.* Aquatic Toxicology, 2015. **165**: p. 101-108.

220. Lürling, M., *Effects of microcystin-free and microcystin-containing strains of the cyanobacterium Microcystis aeruginosa on growth of the grazer Daphnia magna.* Environmental Toxicology, 2003. **18**(3): p. 202-210.

221. Rohrlack, T., E. Dittmann, M. Henning, T. Börner, and J.G. Kohl, *Role of microcystins in poisoning and food ingestion inhibition of Daphnia galeata caused by the cyanobacterium Microcystis aeruginosa.* Applied and Environmental Microbiology, 1999. **65**(2): p. 737-739.

222. Réveillon, D., V. Séchet, P. Hess, and Z. Amzil, *Systematic detection of BMAA (β-N-methylamino-L-alanine) and DAB (2,4-diaminobutyric acid) in mollusks collected in shellfish production areas along the French coasts.* Toxicon, 2016. **110**: p. 35-46.

223. Salomonsson, M.L., E. Fredriksson, A. Alfjorden, M. Hedeland, and U. Bondesson, *Seafood sold in Sweden contains BMAA: A study of free and total concentrations with UHPLC-MS/MS and dansyl chloride derivatisation.* Toxicology Reports, 2015. **2**: p. 1473-1481.

224. O'Neil, J.M., T.W. Davis, M.A. Burford, and C.J. Gobler, *The rise of harmful cyanobacteria blooms: The potential roles of eutrophication and climate change.* Harmful Algae, 2012. **14**: p. 313-334.

225. Kosten, S., V.L.M. Huszar, E. Bécares, L.S. Costa, E. van Donk, L.A. Hansson, E. Jeppesen, C. Kruk, G. Lacerot, N. Mazzeo, L. De Meester, B. Moss, M. Lürling, T. Nõges, S. Romo, and M. Scheffer, *Warmer climates boost cyanobacterial dominance in shallow lakes.* Global Change Biology, 2012. **18**(1): p. 118-126.

226. Hinder, S.L., G.C. Hays, M. Edwards, E.C. Roberts, A.W. Walne, and M.B. Gravenor, *Changes in marine dinoflagellate and diatom abundance under climate change.* Nature Climate Change, 2012. **2**(4): p. 271-275.

227. Hallegraeff, G.M., *Ocean climate change, phytoplankton community responses, and harmful algal blooms: A formidable predictive challenge.* Journal of Phycology, 2010. **46**(2): p. 220-235.

228. Vézie, C., J. Rapala, J. Vaitomaa, J. Seitsonen, and K. Sivonen, *Effect of nitrogen and phosphorus on growth of toxic and nontoxic Microcystis strains and on intracellular microcystin concentrations.* Microbial ecology, 2002. **43**(4): p. 443-454.

229. Tonk, L., D.B. Van De Waal, P. Slot, J. Huisman, H.C.P. Matthijs, and P.M. Visser, *Amino acid availability determines the ratio of microcystin variants in the cyanobacterium Planktothrix agardhii.* FEMS Microbiology Ecology, 2008. **65**(3): p. 383-390.

230. Utkilen, H. and N. Gjolme, *Iron-stimulated toxin production in Microcystis aeruginosa.* Applied and Environmental Microbiology, 1995. **61**(2): p. 797-800.

231. Ger, K.A., E.J. Faassen, M.G. Pennino, and M. Lürling, *Effect of the toxin (microcystin) content of Microcystis on copepod grazing.* Harmful Algae, 2016. **52**: p. 34-45.

"Questions of science; science and progress
Do not speak as loud as my heart"

Coldplay, The Scientist
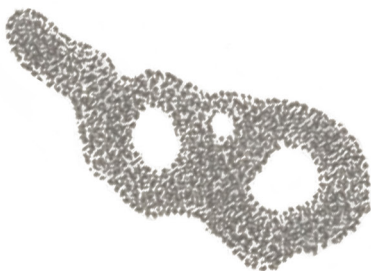
Mama, bedankt dat je me altijd in het goede van mensen hebt laten vertrouwen. Papa, heel erg bedankt dat je er altijd voor ons bent geweest, ook in tijden die ontzettend zwaar voor je moeten zijn geweest. Het is voor een groot deel aan jou te danken dat we zo goed terecht zijn gekomen. Els, bedankt dat je de vreugde weer terug hebt gebracht bij ons thuis, je bent een geweldig mens. Tineke en Leo: we lopen elkaars deuren niet plat, maar ik ben blij met jullie als broer en zus, en Tineke, ik ben trots dat jij als paranimf deze dag naast me staat. Dank ook aan Kees en Hennie. En dan natuurlijk Dennis, mijn rustpunt en onvoorwaardelijke steun. Je bent er altijd voor me, wat ik ook doe, en dat is een heerlijk gevoel. Siemen en Janna, niemand kan mijn leven zo veranderen als jullie dat hebben gedaan. Siemen, je wonderlijke gedachtegangen, knuffels vol overgave en sterke wil maken me erg vrolijk en zorgen ervoor dat er thuis nooit een saai moment is. Janna, met jouw oneindig enthousiasme en goed humeur ben je met recht ons zonnestraaltje, en qua knuffelen doe je niet onder voor je broer! Ik ben gezegend met jullie!

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Elisabeth Johanna Faassen was born in Dommelen, the Netherlands in 1979. In 1997, she passed the gymnasium at Christiaan Huygens College in Eindhoven *cum laude*. In the same year, she started her studies Environmental Sciences at Wageningen University, in which she specialized in Water Quality. For her master theses and internship, she studied thyroid hormone disruption in frogs and cell lines, the foraging behaviour of oystercatchers and denitrification in a ditch system. After receiving her MSc *cum laude* in 2003, she was employed at the local water manager Hoogheemraadschap van Rijnland. Here, she worked as a modeler and ecologist on urban waters and on the Water Framework Directive. In 2008, she returned to the Aquatic Ecology and Water Quality Management Group of Wageningen University as a PhD student, to perform the research presented in this thesis. From November 2015 to June 2016, she held a postdoc position at the Aquatic Ecology team of the Netherlands Institute of Ecology. Els is currently working as experimental researcher ecotoxicology at the Environmental Risk Assessment team of Wageningen UR.

AWARDS

2016: The paper 'Presence of the neurotoxin BMAA in aquatic ecosystems: What do we really know?' was selected as the Dutch submission to the international SIL (the International Society of Limnology) student competition.

2011: Honorable distinction by the Storm-van der Chijs Fund. This distinction is awarded to very promising female PhD candidates of Wageningen University.

PEER REVIEWED PUBLICATIONS

Faassen, E.J., M.G. Antoniou, W. Beekman-Lukassen, L. Blahova, E. Chernova, C. Christophoridis, A. Combes, C. Edwards, J. Fastner, J. Harmsen, A. Hiskia, L.L. Ilag, T. Kaloudis, S. Lopicic, M. Lürling, H. Mazur-Marzec, J. Meriluoto, C. Porojan, Y. Viner-Mozzini, and N. Zguna, *A collaborative evaluation of LC-MS/MS based methods for BMAA analysis: Soluble bound BMAA found to be an important fraction.* Marine Drugs, 2016. **14**(3).

Ger, K.A., **E.J. Faassen**, M.G. Pennino, and M. Lürling, *Effect of the toxin (microcystin) content of Microcystis on copepod grazing*. Harmful Algae, 2016. **52**: p. 34-45.

Liu, J., E. Van Oosterhout, **E.J. Faassen**, M. Lürling, N.R. Helmsing, and D.B. Van de Waal, *Elevated pCO$_2$ causes a shift towards more toxic microcystin variants in nitrogen-limited Microcystis aeruginosa*. FEMS Microbiology Ecology, 2016. **92**(2): p. 1-8.

Rangel, L.M., K.A. Ger, L.H.S. Silva, M.C.S. Soares, **E.J. Faassen**, and M. Lürling, *Toxicity overrides morphology on Cylindrospermopsis raciborskii grazing resistance to the calanoid copepod Eudiaptomus gracilis*. Microbial Ecology, 2016. **71**(4): p. 1-10.

Faassen, E.J., M. García-Altares, M. Mendes e Mello, and M. Lürling, *Trans generational effects of the neurotoxin BMAA on the aquatic grazer Daphnia magna*. Aquatic Toxicology, 2015. **168**: p. 98-107.

Faassen, E.J., A.J. Veraart, E.H. Van Nes, V. Dakos, M. Lürling, and M. Scheffer, *Hysteresis in an experimental phytoplankton population*. Oikos, 2015. **124**(12): p. 1617-1623.

Faassen, E.J., *Presence of the neurotoxin BMAA in aquatic ecosystems: What do we really know?* Toxins, 2014. **6**(3): p. 1109-1138.

Lürling, M., D. Meng, and **E.J. Faassen**, *Effects of hydrogen peroxide and ultrasound on biomass reduction and toxin release in the cyanobacterium, Microcystis aeruginosa*. Toxins, 2014. **6**(12): p. 3260-3280.

Waajen, G.W.A.M., **E.J. Faassen**, and M. Lürling, *Eutrophic urban ponds suffer from cyanobacterial blooms: Dutch examples.* Environmental Science and Pollutions Research, 2014. **21**(16): p. 9983-9994.

Ekvall, M.K., J. de la Calle Martin, **E.J. Faassen**, S. Gustafsson, M. Lürling, and L.A. Hansson, *Synergistic and species-specific effects of climate change and water colour on cyanobacterial toxicity and bloom formation.* Freshwater Biology, 2013. **58**(11): p. 2414-2422.

Faassen, E.J., W. Beekman, and M. Lürling, *Evaluation of a commercial enzyme linked immunosorbent assay (ELISA) for the determination of the neurotoxin BMAA in surface waters.* PLoS ONE, 2013. **8**(6).

Faassen, E.J. and M. Lürling, *Occurrence of the microcystins MC-LW and MC-LF in Dutch surface waters and their contribution to total microcystin toxicity.* Marine Drugs, 2013. **11**(7): p. 2643-2654.

Lürling, M. and **E.J. Faassen**, *Dog poisonings associated with a Microcystis aeruginosa bloom in the Netherlands*. Toxins, 2013. **5**(3): p. 556-567.

Lürling, M., F. Eshetu, **E.J. Faassen**, S. Kosten, and V.L.M. Huszar, *Comparison of cyanobacterial and green algal growth rates at different temperatures*. Freshwater Biology, 2013. **58**(3): p. 552-559.

Faassen, E.J., F. Gillissen, and M. Lürling, *A comparative study on three analytical methods for the determination of the neurotoxin BMAA in cyanobacteria*. PLoS ONE, 2012. **7**(5).

Faassen, E.J., L. Harkema, L. Begeman, and M. Lürling, *First report of (homo)anatoxin-a and dog neurotoxicosis after ingestion of benthic cyanobacteria in The Netherlands*. Toxicon, 2012. **60**(3): p. 378-384.

Lürling, M. and **E.J. Faassen**, *Controlling toxic cyanobacteria: Effects of dredging and phosphorus-binding clay on cyanobacteria and microcystins.* Water Research, 2012. **46**(5): p. 1447-1459.

Veraart, A.J., **E.J. Faassen**, V. Dakos, E.H. Van Nes, M. Lürling, and M. Scheffer, *Recovery rates reflect distance to a tipping point in a living system*. Nature, 2012. **481**(7381): p. 357-359.

Lürling, M., **E.J. Faassen**, and J.S.V. Eenennaam, *Effects of the cyanobacterial neurotoxin β-N-methylamino-L-alanine (BMAA) on the survival, mobility and reproduction of Daphnia magna*. Journal of Plankton Research, 2011. **33**(2): p. 333-342.

Faassen, E.J., F. Gillissen, H.A.J. Zweers, and M. Lürling, *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*. Amyotrophic Lateral Sclerosis, 2009. **10**(SUPPL. 2): p. 79-84.

Schriks, M., M.K. van Hoorn, **E.J. Faassen**, J.W. van Dam, and A.J. Murk, *Real-time automated measurement of Xenopus leavis tadpole behavior and behavioral responses following triphenyltin exposure using the multispecies freshwater biomonitor (MFB)*. Aquatic Toxicology, 2006. **77**(3): p. 298-305.

Schriks, M., C.M. Vrabie, A.C. Gutleb, **E.J. Faassen**, I.M.C.M. Rietjens, and A.J. Murk, *T-screen to quantify functional potentiating, antagonistic and thyroid hormone-like activities of poly halogenated aromatic hydrocarbons (PHAHs)*. Toxicology In Vitro, 2006. **20**(4): p. 490-498.

BOOK CHAPTER

Faassen, E. J., SOP 22 Extraction and LC-MS/MS analysis of underivatised BMAA. In: *Handbook of Cyanobacterial Monitoring and Cyanotoxin Analysis*, 2016. Edited by J. Meriluoto, L. Spoof, and G.A. Codd. John Wiley & Sons, Chichester, UK. *In press.*

OTHER PUBLICATIONS

Faassen, E.J., L.N. De Senerpont Domis, D.B. Van de Waal, and M. Lürling, *Commentary: The dark side of the Bloom*. Science, 2014.

Geurts, J., L.N. De Senerpont Domis, M. Lürling, M. and **E. Faassen,** *Onderzoek naar blauwalgenproblematiek in zwemwateren in de Alblasserwaard.* B-ware/AKWA report, 2013.

Kosten, S., E. Kardinaal, **E. Faassen**, J. Netten, and M. Lürling, *Klimaat & waterkwaliteit, Klimaatinvloed op waterkwaliteit en het voorkomen van cyanobacteriële toxines.* Kennis voor Klimaat report, 2011.

Faassen, E. and M. Lurling, *Inventarisatie van cyanotoxines in Nederlands oppervlaktewater.* WUR report, 2010.

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Wageningen, 8 July 2016

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The SENSE Research School declares that **Ms Els Faassen** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 48.1 EC, including the following activities:

SENSE PhD Courses

o   Life history theory (2009)
o   Environmental Research in Context (2009)
o   Special topics in ecotoxicology: Marine ecotoxicology (2010)
o   Research in Context Activity: 'Water quality advice Terra Nova' (2010)

Other PhD and Advanced MSc Courses

o   Good Laboratory Practice, Wageningen University (2009)
o   Information literacy PhD including Endnote, Wageningen University (2009)
o   Techniques for writing and presenting scientific papers, Wageningen University (2009)
o   Supervising thesis students, Wageningen University (2010)
o   Scientific publishing, Wageningen University (2013)

External training at a foreign research institute

o   Training in LC-MS/MS instruments, Agilent Technologies, Germany (2009)
o   BMAA analysis, Institute for EthnoMedicine, United States (2009)
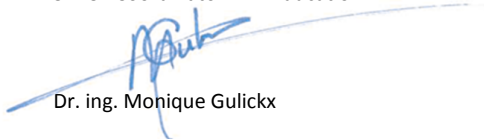o   BMAA analysis, Stockholm University, Sweden (2010)

Management and Didactic Skills Training

o   Supervising four MSc students, Wageningen University (2008-2015)
o   Supervising three BSc students, Wageningen University (2008-2015)
o   Organising and instructing workshop BMAA analysis, initiated by CYANOCOST, Wageningen University (2015)

Selection of Oral Presentations

o   *Underivatised BMAA analysis in Dutch urban waters.* 5th International BMAA Symposium, 13-15 October 2009, Jackson Hole, United States
o   *Comparing analytical methods for BMAA detection.* 8th International Conference on Toxic Cyanobacteria (ICTC8), 29 August-4 September 2010, Istanbul, Turkey
o   *Microcystin production under elevated T and CO_2.* International Limnological Society Congress (SIL XXXII), 4-9 August 2013, Budapest, Hungary
o   *The role of analytical methods in BMAA research.* BMAA–ALS Symposium, 4-5 February 2016, Limoges, France

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