Stockholm University

The neurotoxin β-N-methylamino-L-alanine (BMAA)

Sources, bioaccumulation and extraction procedures

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Cover image: Cyanobacteria, diatoms and dinoflagellates microscopic pictures taken by Sandra Ferreira Lage

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“Sinto mais longe o passado,
sinto a saudade mais perto.”

Fernando Pessoa, 1914.

Abstract

β-methylamino-L-alanine (BMAA) is a neurotoxin linked to neurodegeneration, which is manifested in the devastating human diseases amyotrophic lateral sclerosis, Alzheimer's and Parkinson's disease. This neurotoxin is known to be produced by almost all tested species within the cyanobacterial phylum including free living as well as the symbiotic strains. The global distribution of the BMAA producers ranges from a terrestrial ecosystem on the Island of Guam in the Pacific Ocean to an aquatic ecosystem in Northern Europe, the Baltic Sea, where annually massive surface blooms occur. BMAA had been shown to accumulate in the Baltic Sea food web, with highest levels in the bottom dwelling fish-species as well as in mollusks.

One of the aims of this thesis was to test the bottom-dwelling bioaccumulation hypothesis by using a larger number of samples allowing a statistical evaluation. Hence, a large set of fish individuals from the lake Finjasjön, were caught and the BMAA concentrations in different tissues were related to the season of catching, fish gender, total weight and species. The results reveal that fish total weight and fish species were positively correlated with BMAA concentration in the fish brain. Therefore, significantly higher concentrations of BMAA in the brain were detected in plankti-benthivorous fish species and heavier (potentially older) individuals.

Another goal was to investigate the potential production of BMAA by other phytoplankton organisms. Therefore, diatom cultures were investigated and confirmed to produce BMAA, even in higher concentrations than cyanobacteria. All diatom cultures studied during this thesis work were show to contain BMAA, as well as one dinoflagellate species. This might imply that the environmental spread of BMAA in aquatic ecosystems is even higher than previously thought.

Earlier reports on the concentration of BMAA in different organisms have shown highly variable results and the methods used for quantification have been intensively discussed in the scientific community. In the most recent studies, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become the instrument of choice, due to its high sensitivity and selectivity. Even so, different studies show quite variable concentrations of BMAA. In this thesis, three of the most common BMAA extraction protocols were evaluated in order to find out if the extraction could be one of the sources of variability. It was found that the method involving precipitation of proteins using trichloroacetic acid gave the best performance, complying with all in-house validation criteria. However, extractions of diatom and

cyanobacteria cultures with this validated method and quantified using LC-MS/MS still resulted in variable BMAA concentrations, which suggest that also biological reasons contribute to the discrepancies.

The current knowledge on the environmental factors that can induce or reduce BMAA production is still limited. In cyanobacteria, production of BMAA was earlier shown to be negative correlated with nitrogen availability – both in laboratory cultures as well as in natural populations. Based on this observation, it was suggested that in unicellular non-diazotrophic cyanobacteria, BMAA might take part in nitrogen metabolism. In order to find out if BMAA has a similar role in diatoms, BMAA was added to two diatom species in culture, in concentrations corresponding to those earlier found in the diatoms. The results suggest that BMAA might induce a nitrogen starvation signal in diatoms, as was earlier observed in cyanobacteria. Thus, also in diatoms, BMAA might be involved in the nitrogen balance in the cell.

Sammanfattning

β-metylamino-L-alanin (BMAA) är ett neurotoxin som orsakar neurodegeneration och är kopplat till förödande neurologiska sjukdomar som amyotrofisk lateralskleros, Alzheimers och Parkinsons sjukdomar. BMAA produceras av nästan alla analyserade cyanobakteriearter – från de som lever fritt till de som lever i symbiotiska relationer. Dessa BMAA-producenter förekommer globalt och har hittats i åtskilda olika ekosystem världen över – från det terrestra ekosystemet på ön Guam i Stilla havet till det bräckta akvatiska ekosystemet i Östersjön där massiva cyanobakterieblomningar årligen förekommer. Innan detta avhandlingsarbete startade hade det visats att BMAA kan ackumuleras i Östersjön näringsväv med de högsta nivåerna i de bottenlevande fiskarterna samt i vattenfiltrerande mollusker, som tex musslor och ostron.

Ett av syftena med denna avhandling var att pröva hypotesen om ackumulering av BMAA - särskilt i bottenlevande fiskarter - med ett tillräckligt antal prover för att kunna utföra en statistisk analys. Ett stort antal fiskindivider fångades från en relativt liten sjö, Finjasjön och innehållet av BMAA i olika vävnader relaterades till fångstsäsong, kön, totalvikt och art. Resultaten visade en positiv korrelation mellan faktorernas födomönster (planktonätande och bottenlevande) samt totalvikt med höga koncentrationer av BMAA i fiskarnas hjärna.

Ett annat mål med avhandlingen var att undersöka om andra grupper av växtplankton än cyanobakterier har förmåga att producera BMAA. Genom att undersöka ett antal kulturer med kiselalger samt även dinoflagellater kunde vi påvisa att båda dessa grupper har förmåga att producera BMAA samt att de producerar högre nivåer av BMAA än cyanobakterier. Dessa resultat indikerar att effekten av BMAA i vårt akvatiska ekosystem kan vara ännu högre än man tidigare trott.

Tidigare studier har visat stora variationer i koncentrationerna av BMAA och det har lett till intensiva diskussioner angående de metoder som används för att bestämma halterna. Flertalet nyare publikationer använder vätskekromatografi- tandem masspektrometri (LC-MS/MS), baserat på hög känslighet och selektivitet. Trots detta kvarstår problematiken med stora skillnader i koncentrationen av BMAA mellan olika studier. I denna avhandling har jag studerat de tre vanligaste extraktionsmetoderna av BMAA och utvärderat om extraktionen kan vara en av anledningarna till den stora variationen.

Resultaten visar att den metod där triklorättiksyra används för att fälla ut proteiner i provet gav det bästa resultatet och uppfyller samtliga kriterier för metodvalidering.

Den nuvarande kunskapen om vilka miljöfaktorer som kan inducera eller minska ackumuleringen av BMAA i producenterna är fortfarande begränsad. Produktionen av BMAA hos cyanobakterier har visat sig vara negativt korrelerad med tillgången av kväve - både i laboratoriekulturer och i naturliga populationer. Baserat på denna observation har det föreslagits att BMAA i encelliga icke- kvävefixerande cyanobakterier kan vara involverad i kväveomsättningen. För att undersöka om BMAA skulle kunna ha en liknande roll i kiselalger, har jag tillsatt BMAA till kulturer med två arter av kiselalger i koncentrationer som motsvarar de som jag funnit i kiselalgerna. Resultaten visar att BMAA tas upp av kiselalgerna och att de reagerar på liknande sätt som vid kvävebrist. Följdaktligen så är BMAA med stor sannolikhet även i kiselalger inblandad i den cellulära kvävebalansen.

List of Papers

The following papers, referred to by their Roman numerals, are the basis of this thesis:

I. **Lage, S.**, Annadotter, H., Rasmussen, U., Rydberg, S., (2015). Biotransfer of β-N-methylamino-L-alanine (BMAA) in a eutrophicated freshwater lake. Marine Drugs; 13(3):1185-201.

II. Jiang, L., Eriksson, J., **Lage, S.**, Jonasson, S., Shams, S., Mehine, M., Ilag, L., Rasmussen, U., (2014). Diatoms: a novel source for the neurotoxin BMAA in aquatic environments. PLoS One 9: e84578.

III. **Lage, S.**, Burian, A., Rasmussen, U., Costa, P.R., Annadotter, H., Godhe, A., Rydberg, S., (2015). BMAA extraction of cyanobacteria samples: which method to choose? Environmental Science and Pollution Research; 23(1):338-50.

IV. **Lage, S.**, Ström, L., Godhe, A., Rydberg, S., (2016). The effect of exogenous β-N-methylamino-L-alanine on the diatoms *Phaeodactylum tricornutum* and *Thalassiosira weissflogii.* (manuscript).

My contribution to the papers:

Paper I: Performed the experiments, analyzed the data and was the main writer of the paper.

Paper II: Participated in sample preparation, method development and manuscript writing.

Paper III: Participated in the experimental design. Performed the experiment, developed methods, analyzed the data and was the main writer of the paper.

Paper IV: Participated in the experimental design. Participated in and supervised a student in the experimental execution. Analyzed the data and was the main writer of the paper.

Additional paper completed during the PhD studies:

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

Contents

Abbreviations

AEG	N-(2-aminoethyl) glycine
ALS	amyotrophic lateral sclerosis
ALS/PDC	amyotrophic lateral sclerosis/parkinsonism-dementia complex
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
BAMA	β-amino-N-methyl-alanine
BMAA	β-N-methylamino-L-alanine
CCAP	Culture Collection of Algae and Protozoa
CE	capillary electrophoresis
CID	collision induced dissociation
CNS	central nervous system
D_3	deuterated
DAB	2,4-diaminobutyric acid
DABA	2,3-diaminobutyric acid
DNS	dansyl chloride derivatization
DW	dry weight
ELISA	enzyme – linked immunosorbent
ESI	electrospray ionization
FMOC	9-fluorenylmethyl chloroformate
GC-MS	gas chromatography – mass spectrometry
GOGAT	glutamate synthase
GS	glutamine synthetase
GUMACC	Gothenburg University's Marine Algal Culture Collection
HILIC	hydrophilic interaction liquid chromatography
HPLC-FLD	high performance liquid chromatography – fluorescence detection
LC	liquid chromatography
LC-MS	liquid chromatography – mass spectrometry
LC-MS/MS	liquid chromatography –tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MeOH	methanol
mGluRs	metabotropic glutamate receptors
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
NMDA	N-methyl-D-aspartate

nr	number
PCC	The Pasteur Culture Collection of Cyanobacteria
pK_a	acid dissociation constant at logarithmic scale
Q_1	first quadrupole
Q_2	second quadrupole/collision cell
Q_3	third quadrupole
ROS	reactive oxygen species
SCCAP	The Scandinavian Culture Collection of Algae and Protozoa
S/N	signal-to-noise ratio
sp.	species (singular)
SPE	solid phase extraction
spp.	species (plural)
SRM	selective reaction monitoring
TCA	trichloroacetic acid
UPLC	ultra-performance liquid chromatography
Xc^-	cystine/glutamate antiporter system
WW	wet weight

Introduction

BMAA: a neurotoxic non-protein amino acid

β-N-methylamino-L-alanine (BMAA), a neurotoxic non-protein amino acid, also named L-α-amino-β-methylaminopropionic acid, 2-amino-3-(methylamino)-propionic acid or β-diaminopropionic acid, has the molecular formula $C_4H_{10}N_2O_2$ (CAS registry no: 15920-93-1 for L-BMAA hydrochloride) (Fig. 1), and a molecular weight of 118.13 $g \cdot mol^{-1}$. BMAA has a carboxyl group with pKa_1 2.1 and two amino groups,with pKa_2 6.5 and pKa_3 9.8, for primary and secondary amine, respectively (Nunn and O'Brien, 1989). Thus, BMAA is at equilibrium at pH 7, i.e. a zwitter ion, and accepts a proton when it is in lower pH, and donates a proton at higher pH (Nunn and Ponnusamy, 2009).

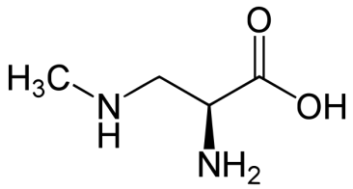

Figure 1. Chemical structure of β-N-methylamino-L-alanine

Although BMAA has several theoretical structural isomers, only seven can be commonly observed with techniques applied to BMAA detection (Jiang et al., 2012). These isomers are 2,4-diaminobutyric acid (DAB), N-(2-aminoethyl) glycine (AEG), β-amino-N-methylalanine (BAMA), 2,3-diaminobutyric acid (DABA), 3,4-diaminobutyric acid, 3-amino-2-(aminomethyl)-propanoic acid and 2,3-diamino-2-methylpropanoic acid (Jiang et al., 2012). DAB, has also shown to be neurotoxic, although primarily hepatotoxic (O'Neal et al., 1968); AEG has been hypothesized to be backbone of the peptide nucleic acids which facilitated the transmission of genetic information during the pre-RNA world (Nelson et al., 2000). The potential function of the other isomers is still unknown and the study of these isomers in the context of BMAA is limited.

BMAA as well as all other non-protein amino acids does not belong to the 22-amino acid group that is incorporated into the structure of proteins on ribosomes, according to the sequence of nucleotides in mRNA. Usually, non-protein amino acids are vari-

ations of protein amino acids. These changes occur through the alteration of the amino to carboxyl relative position and/or the extent of the alkyl chain. Substitutions along the alkyl chain, on the amino group, on any other additional functional group and/or chiral carbons in the R-chain can also generate non-protein amino acids (Pizzarello, 2015). BMAA itself is a variation of alanine (Brenner et al., 2003). Many amino acids have the ability to form two different enantiomers around the central carbon, which leads to the two different isomers, the L and the D-form. This common feature is also applied to BMAA; however, in general, it is only the L form that is incorporated into proteins (Bell, 2009; Dunlop et al., 2013; Glover et al., 2014).

Fungi, bacteria, and plants can produce non-protein amino acids. These compounds are known to be metabolites or intermediates in various metabolisms or part of the biological structures. When consumed by other organisms several of these substances are toxic through the inhibition or disruption of existing proteins (Bell, 2003; Harada, 2004; Vranova et al., 2011). Misincorporation of any of the 22 standard amino acids at error rates as low as 1/10 000 may lead to neurodegeneration in laboratory animals (Lee et al., 2006). L-BMAA is also able to misincorporate into human proteins and subsequently cause protein misfolding, aggregation and/or loss of function (Dunlop et al., 2013; Glover et al., 2014). The misincorporation of BMAA into proteins has earlier been proposed as a mechanism for bioaccumulation as well as a mechanism for a slow release of BMAA within the central nervous system (CNS) (Dunlop et al., 2013). However, this process could be reversed by the presence of L-serine (Dunlop et al., 2013). Just as many other non-protein amino acids, BMAA is not constantly present in a free form; it may also be associated with, bound to, or incorporated into proteins (Murch et al., 2004a; Rodgers and Shiozawa, 2008; Bell, 2009; Cheng and Banack, 2009).

BMAA and the paralytic disease among Guam indigenes

BMAA has been linked to the fatal neurodegenerative diseases, amyotrophic lateral sclerosis (ALS), Parkinson's and Alzheimer's disease (Banack and Cox, 2003a; Murch et al., 2004b; Cox et al., 2009; Pablo et al., 2009). The neurotoxicity of BMAA was discovered half a century ago, following studies conducted on the island of Guam in the Western Pacific Ocean, where patients showed pathologies characteristic of both ALS and Parkinson's disease, i.e. amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS/PDC). The frequency of the disease was abnormally high among the indigenous population of Guam, the so called Chamorro (Vega and Bell, 1967; Spencer et al., 1987b; Spencer et al., 1991).

BMAA was later proposed to be the environmental agent causing the exceptional high incidences of ALS/PDC among the Chamorro population (Vega and Bell, 1967; Spencer et al., 1987b; Spencer et al., 1991). The compound, BMAA, was later

found throughout the tissues of the cycad tree *Cycas circinalis* (now *Cycas micronesica*. Hill), and was particularly abundant in the cycad seeds and immature pollen (Vega and Bell, 1967; Hill, 1994; Banack and Cox, 2003b). An etiological study discovered that the cycad seeds were commonly used as food source by the Chamorro, who made flour from the seeds and prepared tortillas (Whiting, 1988). Thus, the cycad seeds were proposed as the BMAA source (Vega and Bell, 1967; Spencer et al., 1987b). However, later on, only low concentrations of BMAA were detected in the cycad flour (Duncan et al., 1988; Duncan et al., 1989); at least too low to be the likely cause of any neurological effect in the Chamorro (Garruto et al., 1988; Duncan et al., 1990). Consequently, the hypothesis of the link between BMAA and ALS/PDC in Guam was at this point abandoned.

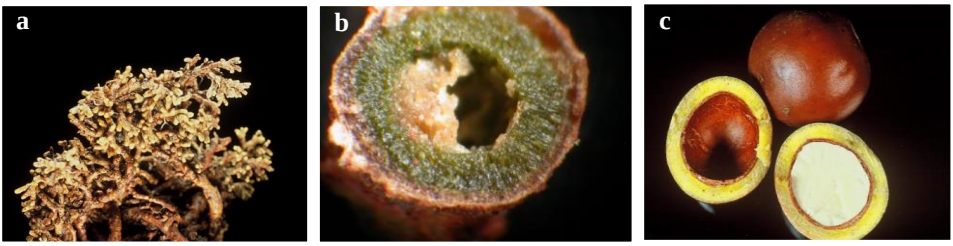

Figure 2. A photo of (a) cycad *Cycas micronesica* coralloid roots, (b) cyanobacteria *Nostoc sp*. in the cycad coralloid roots and (c) *Cycas micronesica* seeds. Pictures credits: Paul Cox, Patty Stewart, and Sandra Banack.

The interest in BMAA was brought back by the beginning of the 21st century, when a revolutionizing study brought new facts to the story (Murch et al., 2004a). The source of BMAA was traced to cyanobacteria of the genus *Nostoc*, which live symbiotically in the coralloid roots of cycads (Fig. 2b) and BMAA was suggested to be then possibly transferred to the cycad seeds (Fig. 2c) (Banack and Cox, 2003b; Cox et al., 2003; Murch et al., 2004a). The concentration of BMAA in the protein-associated fraction was found to be much higher than the BMAA found in the free form (Fig. 3) (Murch et al., 2004a). The protein fraction was not taken into account in the earlier studies (Spencer et al., 1987b; Duncan et al., 1988; Whiting, 1988; Duncan et al., 1989) due to a lack of knowledge and selectivity of the instrumentation used. Added to this, both free BMAA and protein associated BMAA were then proven to accumulate in both cycad seeds and the wild animals (e.g. flying foxes, pigs and deer) that forage on them (Fig. 3) (Murch et al., 2004a).

These new data could now explain how the neurotoxin BMAA had reached the indigenous Chamorro population through biomagnification, i.e. an increasing accumulation of a molecule through higher trophic levels (Fig. 3) (Banack and Cox, 2003a; Cox et al., 2003; Murch et al., 2004a). The diet of the Chamorros comprised several wild animals in particular the flying foxes, which feed almost exclusively on cycad seeds and evidently accumulate BMAA in high concentrations in their body parts (Fig. 3) (Banack and Cox, 2003a; Banack et al., 2006). To further ensure

BMAA as the causative agent of ALS/PDC development in the Chamorro, BMAA was detected in *post-mortem* brain tissues of patients suffering from ALS/PDC (Cox et al., 2003; Murch et al., 2004b).

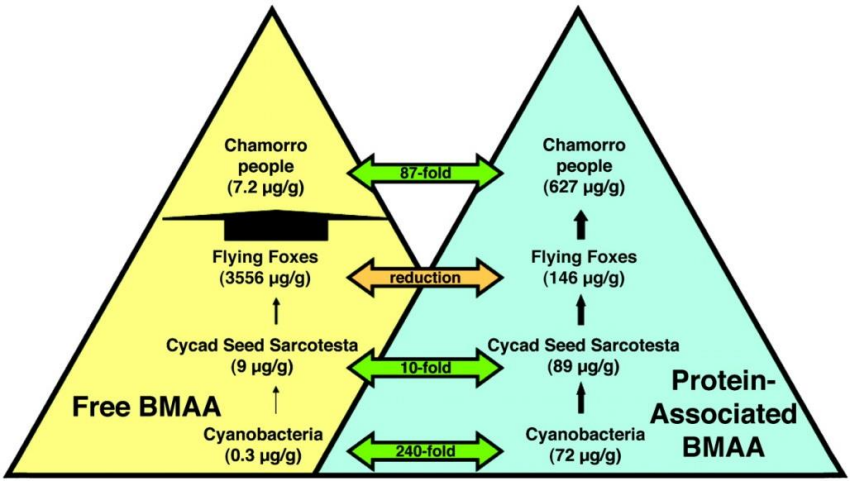

Figure 3. Illustration of the bioaccumulation of BMAA (free and protein-bound) in the Guam ecosystem, from Murch, S.J., Cox, P.A., Banack, S.A., 2004a. A mechanism for slow release of biomagnified cyanobacterial neurotoxins and neurodegenerative disease in Guam. Proceedings of the National Academy of Sciences 101, 12228-12231; Copyright © 2004, The National Academy of Sciences.

These new findings suggested BMAA as an environmental factor causing neurodegeneration in the Chamorro population in Guam (Cox et al., 2003; Murch et al., 2004b). Consequently, this discovery generated a worldwide interest in the subject, especially due to the global distribution of the producer (i.e. cyanobacteria). BMAA, together with several other environmental compounds is currently thought to be implicated in the development of sporadic ALS, which corresponds to 90–92% of all cases worldwide (Banack et al., 2010a; Logroscino et al., 2010). In fact, BMAA has been detected in *post-mortem* brain and spinal cord tissues of ALS, Alzheimer's and Parkinson's patients living geographically far from Guam (Bradley and Cox, 2009; Bradley and Mash, 2009; Pablo et al., 2009).

Moreover, BMAA has shown to bioaccumulate in the Baltic Sea food web, i.e. cyanobacteria, zooplankton and several fish species, with highest levels in the bottom dwelling fish species, where several fish tissues contained up to 200 times higher concentrations of BMAA than the cyanobacteria, Fig. 4 (Jonasson et al., 2010). Mussels (*Mytilus edulis*) and oysters (*Ostrea edulis*) cultured on the Swedish West Coast destined for human consumption, also contained relatively high concentrations of BMAA (Jonasson et al., 2010). This was unexpected, since this coastal zone does not suffer from the same pronounced cyanobacteria surface blooms as in the Baltic Proper.

The results obtained in the Baltic Sea study revealed, for the first time, that BMAA was biotransferred in an aquatic ecosystem outside of Guam. Later, other comparable studies conducted in the subtropical, aquatic ecosystem of Florida, USA; in Gonghu Bay, on Lake Taihu, China and Lake Finjasjön in Sweden, confirm the same pattern of BMAA bioaccumulation, with highest level of BMAA in filter feeding organisms and bottom dwelling fish species (Brand et al., 2010; Jiao et al., 2014 ; **Paper I**).

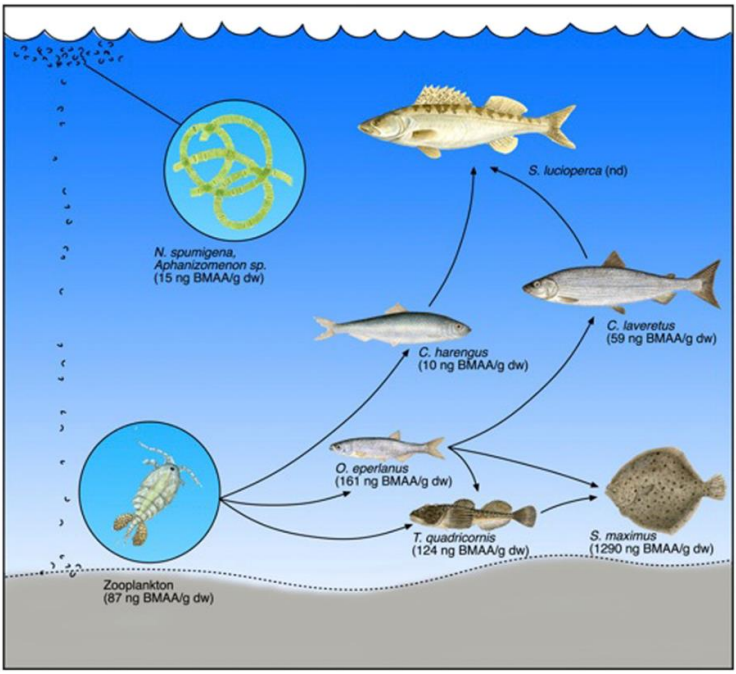

Figure 4. Illustration of BMAA biotransfer in the Baltic Sea; from cyanobacteria to zooplankton and to fishes. Note: BMAA levels indicated represent the highest concentration found in each species; for *T. quadricornis, S. maximus*, and O. *eperlanus* were found in brain tissue, whereas those indicated for *C. harengus* and *C. lavaretus* were found in muscle tissue. ND, not detectable; from Jonasson, S., Eriksson, J., Berntzon, L., Spacil, Z., Ilag, L.L., Ronnevi, L.O., Rasmussen, U., Bergman, B., 2010. Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure. Proceedings of the National Academy of Sciences 107, 9252-9257.

Mechanisms of BMAA neurotoxicity and neurodegeneration

Most toxins produced by phytoplankton cause an acute toxicity, i.e. they possess a short-term poisoning potential - however, this is not applicable for the mechanism of action of BMAA. The neurological effects caused by BMAA can only be observed after long-term exposure, which cause a challenge in understanding the effects of BMAA exposure in humans (Spencer et al., 1987a; Spencer et al., 1991; Karamyan and Speth, 2008). Nevertheless, the BMAA neurotoxicity has been proved in human derived neurons through different pathways, for instance, activation of the N-methyl-D-aspartate (NMDA) receptor at mM concentrations and at µM concentra-

tions in the two metabotropic glutamate receptors 1 and 5 (mGluR1 and mGluR5 receptors, respectively), as well as in the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid (AMPA)/kainate receptors (Lindstrom et al., 1990; Rao et al., 2006; Lobner et al., 2007; Liu et al., 2009a; Liu et al., 2009b). Toxicity studies with BMAA have shown damage both to neuron cultures and to the development of neurodegenerative effects in animals (Karamyan and Speth, 2008; Chiu et al., 2011). For instance, when rats were injected intraperitoneal with 6–14 μm BMAA/g body weight, they showed weakness, convulsions and incoordination (Vega and Bell, 1967). In monkeys fed for 10 weeks with 100–350 mg/kg BMAA, corticomotoneuronal dysfunction, Parkinsonian features and behavioral abnormalities were found (Spencer et al., 1987a). In more recent studies, lower concentrations of BMAA have been used and verified that BMAA can induce long-term cognitive deficits as well as protein changes and fibril formation in the hippocampus of adult rodents following neonatal exposure (Karlsson et al., 2009; Karlsson et al., 2011; Karlsson et al., 2012).

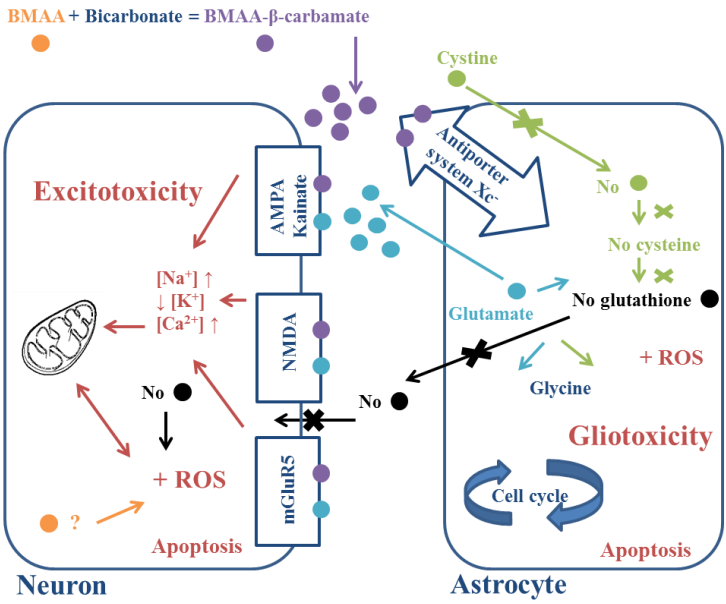

Figure 5. Schematic summary of the toxic mechanisms reported for BMAA *in vitro*; based on Chiu et al. (2011). BMAA in contact with bicarbonate forms BMAA-β-carbamate; which can bind to different glutamate receptors (AMPA/Kainate, NMDA, mGluR5), resulting in prolonged neuron depolarization leading to increase of membrane permeability. Increase of the intracellular Ca^{2+} disrupts cellular homeostasis (exitotoxicity). Presence of ROS in motor neurons is most likely a side effect of the Ca^{2+}entrance. High intracellular $Ca^{2+}$and ROS disrupt mitochondrial activity which will induce more ROS and apoptosis. ROS can also be formed by only BMAA (i.e. without carbamate adduct); however, the mechanism is still unknown. BMAA inhibition of the antiporter system Xc^- results in the glutamate release in the extracellular medium, which will bind to receptors and potentiate excitotoxicity; and inhibition of cystine entering the cell leading to the inhibition of glutathione formation, which indirectly generates ROS. BMAA may also stop the cell cycle at the G2/M phase causing cell apoptosis. The overall increase in the gliotoxicity increases the neuros susceptibility to excitotoxins as BMAA.

BMAA is capable of forming two carbamate adducts in the presence of carbon dioxide/bicarbonate. The formed adducts, α-N-carboxy and β-N-carboxy, are structurally similar to glutamate, the most important transmitter for normal brain functions in mammals (Nunn and O'Brien, 1989; Weiss et al., 1989a). Hence, it is not surprising, that BMAA mechanism of action involves the excess activation of glutamate receptors, which subsequently leads to accumulation of intracellular Ca^{2+} and the generation of reactive oxygen species (ROS). This ultimately leads to destruction of the neurons via an excitotoxic mechanism, i.e. neuronal damaged caused by high concentrations of extracellular glutamate (Nunn et al., 1987; Weiss et al., 1989b; Myers and Nelson, 1990). The effects of BMAA in motor neurons have shown to be far greater than in any other neuron cell, this through the activation of the AMPA/kainate receptor (Rao et al., 2006). In the AMPA/kainate receptor, BMAA induces an excess activation which results in neuron degeneration; via rapid Ca^{2+} entry through the AMPA/kainate channel generating mitochondrial ROS (Fig. 5) (Rao et al., 2006).

In addition to the oxidative stress caused by the BMAA excess activation of the glutamate receptors, BMAA also inhibit the cystine/glutamate antiporter system Xc^- in astrocytes (Liu et al., 2009a). The Xc^- antiporter system is responsible for the transport of cystine into the cell in exchange for glutamate being transported out; thus, the presence of BMAA leads to an increase of extracellular glutamate while glutathione is depleted inside the astrocyte. This mechanistic action will further increase ROS causing further excitotoxicity (Fig. 5) (Liu et al., 2009a). In some of the reported cases of sporadic forms of ALS, a decrease in the glutamate uptake capacity in the spinal cord and motor cortex was noticed. This is likely due to an increase in levels of extracellular glutamate which results in excitotoxicity (Rothstein et al., 1990; Rothstein et al., 1992; Shaw, 1994; Rothstein, 1996). Also in healthy human neurons, the exposure to BMAA causes an increased intracellular Ca^{2+} influx, DNA damage and mitochondrial activity; release of lactate dehydrogenase and generation of ROS, all characteristics of excitotoxicity (Chiu et al., 2012).

Although the BMAA neurotoxicity has been shown both in *in vitro* and in biological models (Karamyan and Speth, 2008; Chiu et al., 2011), as well as in *post-mortem* brain tissue from patients suffering of neurological diseases (Banack and Cox, 2003a; Cox et al., 2003; Murch et al., 2004b; Pablo et al., 2009), proof of the neurodegenerative characteristics via dietary exposure has not been presented until just recently (Cox et al., 2016). In this study, vervet monkeys (*Chlorocebus sabaeus*) were fed for 140 days with fruit supplemented with BMAA. The diet (i.e. 21 mg $kg^{-1}d^{-1}$ of BMAA) was projected to be equivalent to a cumulative life-time exposure of BMAA by a Chamorro, leading to the development of neurofibrillary tangles and β-amyloid deposits in the brains - both neurological hallmarks of Alzheimer's disease and ALS/PDC (Cox et al., 2016).

Cyanobacteria – BMAA producers

Cyanobacteria, previously known as blue-green algae, are included in a highly diverse group of ancient gram-negative photosynthetic prokaryotes, i.e. the Eubacteria kingdom, exhibiting oxygenic photosynthesis. They are extremely important primary producers and constitute the first level of organisms in the globally distributed aquatic food webs (Herrero and Flores, 2008). Moreover, cyanobacteria have the ability to perform anaerobic metabolism and the capacity to use elemental sulfur for anaerobic dark respiration (Cohen and Gurevitz, 2006). They also have an important role in the marine nitrogen cycle, by fixing atmospheric nitrogen (N), and in the global carbon cycle by their photosynthetic activity (Whitton and Potts, 2000). So far, 150 genera and about 2000 species of cyanobacteria have been described (Van Apeldoorn et al., 2007).

In terms of morphology, cyanobacteria are either single celled, colonial or filamentous (Duy et al., 2000). Cyanobacteria exhibit versatile physiology, a wide ecological tolerance and high genetic diversity, which together contributes to their competitive success over a broad spectrum of environments across all global latitudes, demonstrating the ability of the pioneering ancestors as the earliest inhabitants of Earth (Cohen and Gurevitz, 2006). In fact, they inhabit ice fields, hot springs, deserts and are especially common in freshwater, brackish and marine environments (Whitton and Potts, 2000; Cohen and Gurevitz, 2006). In view of the aquatic environments, it is impossible to fully separate freshwater and marine cyanobacteria species, considering that some species are capable to grown in both environments (Burja et al., 2001).

With regard to the ability to produce BMAA, it has been recorded in a wide range of cyanobacteria species, with verified occurrence around the world (Table 1). This includes the cyanobacterial species that annually form the massive surface ‘blooms’ in the Baltic Sea during summer i.e. genera *Nodularia* and *Aphanizomenon* (Cox et al., 2005; Jonasson et al., 2010).

BMAA detection in cyanobacteria: the controversies

Even though BMAA has concurrently been detected in cyanobacterial species from different environments around the world, the levels of BMAA detected have varied considerably (Table 1) (Cox et al., 2005; Esterhuizen and Downing, 2008; Metcalf et al., 2008; Craighead et al., 2009; Faassen et al., 2009; Jonasson et al., 2010; Li et al., 2010). The first report showing BMAA in cyanobacteria in 2005 was alarming, since almost all investigated laboratory grown species, free living as well as symbiotic strains, contained high concentrations of BMAA (Cox et al., 2005). However, these first results could not be reproduced by subsequent studies; BMAA could either not be detected in cyanobacteria (Rosén and Hellenäs, 2008; Kruger et al.,

2010) or detected in some, but not all, (Faassen et al., 2009) or detected in all samples, but at very low concentrations (Jonasson et al., 2010) (Table 1). At first, biological reasons, such as sample origin and growth conditions of laboratory strains, were suggested as the main cause of these discrepancies (Banack et al., 2010a; Banack et al., 2010b). However, a more plausible reason was the diversity of the analytical techniques applied by the different research groups and used between studies (Faassen et al., 2012; Faassen, 2014).

Highest BMAA concentrations and percentages of detected positives samples were found in studies using high performance liquid chromatography with fluorescence detection (HPLC-FLD), gas chromatography with mass spectrometry detection (GC-MS) and capillary electrophoresis (CE) for quantification (Cox et al., 2005; Esterhuizen and Downing, 2008; Metcalf et al., 2008; Baptista et al., 2011). Whereas studies using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) either did not detect BMAA or reported low BMAA concentrations (Rosén and Hellenäs, 2008; Faassen et al., 2009; Jonasson et al., 2010; Kruger et al., 2010). This hypothesis was verified by concurrently analyzing cyanobacteria samples with both the analytical methods HPLC-FLD and LC-MS/MS (Faassen et al., 2012). The HPLC-FLD was proven to be less selective than the LC-MS/MS, this since the only selection criteria used are the retention time and signal fluorescence (Faassen et al., 2012). Thus, HPLC-FLD is an uncertain method when it comes to BMAA detection in complex biological matrices and may most certainly lead to overestimations of the BMAA concentrations. Instead, LC-MS/MS has been shown to give a reliable identification based on the several selection criteria, i.e. retention time, mass-to-charge ratio (*m/z*) of the precursor ion, *m/z* of the product fragment ions after collision-induced dissociation, and the ratio between the intensities of respective ions transitions in MRM spectrum (Faassen et al., 2012; Jiang et al., 2012).

In the more recent studies, BMAA analysis are most often performed with LC-MS/MS (Faassen, 2014). However, even using this analytical method, there are inconsistencies in the concentrations of BMAA reported between studies (Banack et al., 2007; Jonasson et al., 2010; Spacil et al., 2010; Banack et al., 2011; Jiao et al., 2014; Lage et al., 2014 ; **Paper I**; **II** and **III**). It is possible that these differences are due to variation in the protocols for the extraction of BMAA, but it may also partly be due to by biological causes (see **Paper III** and **IV**).

Table 1. Summary of BMAA quantification in cyanobacteria [(1)]

				BMAA concentration (μg.g^{-1}DW)			
Samples origin	**Extraction Method**	**Derivatization Method**	**Analytical Method**	**Free form**	**Protein associated**	**Total**	**References**
Worldwide lab cultures	TCA with HCl hydrolysis	AQC	HPLC-FLD	3-6478	4-5415	n.a.	(Cox et al., 2005)
South Africa	TCA with HCl hydrolysis	EZ:faast ™	GC-MS	n.q.	0.1-2756	n.a.	(Esterhuizen and Downing, 2008)
Great Britain	TCA with HCl hydrolysis	AQC	HPLC- FLD	2-276	6-48	n.a.	(Metcalf et al., 2008)
Peru	TCA with HCl hydrolysis	AQC	HPLC-FD, amino acid analyzer, ULPC-MS and LC-MS/MS	n.a.	n.a.	2-22	(Johnson et al., 2008)
Lab cultures and Baltic sea	TCA with HCl hydrolysis	Underivatized	LC-MS/MS	n.d.	n.d.	n.a.	(Rosén and Hellenäs, 2008)
USA	TCA with HCl hydrolysis	FMOC	HPLC-FLD	n.d.	n.d.	n.a.	(Scott et al., 2009)
Gobi desert	HCl hydrolysis	AQC	LC-MS/MS	n.a.	n.a	n.q.	(Craighead et al., 2009)
Netherlands	TCA with HCl hydrolysis	Underivatized	LC-MS/MS	4-42	n.d.	n.a.	(Faassen et al., 2009)
China	HCl hydrolysis	AQC	LC-MS	n.a.	n.a.	0.027-0.659	(Roney et al., 2009)
Lab cultures	TCA with HCl hydrolysis	Underivatized and AQC	LC-MS and LC-MS/MS	n.a.	n.a.	n.q.	(Li et al., 2010)
Lab cultures	TCA with HCl hydrolysis	Underivatized	LC-MS/MS	n.a.	n.a.	n.d.	(Kruger et al., 2010)
Baltic Sea	80% MeOH with HCl hydrolysis	AQC	LC-MS/MS	n.a.	n.a.	0.001-0.015	(Jonasson et al., 2010)
South Africa	TCA with HCl hydrolysis	EZ:faast ™	LC-MS	0.05-0.976	0.0518-10.616	n.a.	(Esterhuizen-Londt and Downing, 2011)

Portugal	TCA with HCl hydrolysis	Underivatized	CE	n.a.	n.a.	170-810	(Baptista et al., 2011)
Portuguese lab cultures	TCA with HCl hydrolysis and 80% MeOH with HCl Hydrolysis	AQC	HPLC- FLD	n.a.	n.a.	0.04-63	(Cianca et al., 2012)
Lab cultures	75% MeOH with HCl hydrolysis	Underivatized	LC-MS/MS	n.d.	n.d.	n.a.	(Li et al., 2012)
Lab cultures	TCA with HCl hydrolysis	Underivatized and AQC	HPLC-FLD and LC-MS/MS	(2)	n.d.	n.a.	(Faassen et al., 2012)
Netherlands	TCA with HCl hydrolysis	Underivatized	ELISA	(3)	(3)	n.a.	(Faassen et al., 2013)
Spirulina samples	HCl hydrolysis	Underivatized and AQC	LC-MS/MS	n.a.	n.a.	n.d.	(McCarron et al., 2014)
Lab cultures	TCA with HCl hydrolysis	Underivatized	LC-MS/MS	n.d.	n.d.	n.a.	(Reveillon et al., 2014)
Lake Taihu, China	TCA with HCl hydrolysis	AQC	LC-MS/MS	n.r.	n.r.	2.01-7.23	(Jiao et al., 2014)
Nebraska, USA	TCA with HCl hydrolysis	AQC	HPLC-FLD	n.a.	n.a.	1.8-25.3 (4)	(Al-Sammak et al., 2014)
Al Kharrara, Qatar	HCl hydrolysis	AQC	LC-MS/MS	n.a.	n.a.	n.d.	(Richer et al., 2015)
Québec, Canada	MeOH	DNS	LC-MS/MS	0.01-0.3 (4)	n.a.	n.a.	(Roy-Lachapelle et al., 2015)

TCA: Trichloroacetic acid; HCl: Hydrochloric acid; MeOH: Methanol; AQC: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; EZ:faast ™: propyl chloroformate; FMOC: 9-fluorenylmethyl chloroformate; DNS: Dansyl chloride derivatization; HPLC-FLD: high performance liquid chromatography with fluorescence detection; GC-MS: Gas chromatography–mass spectrometry; ULPC-MS: Ultra-performance liquid chromatography- mass spectrometry; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; LC-MS: Liquid chromatography–mass spectrometry; CE: Capillary electrophoresis; ELISA: Enzyme-Linked Immunosorbent Assay; n.a.: not analyzed; n.d.: not detectable; n.q.: detectable but not quantifiable; n.r.: not reported; (1) The table only presents studies in which at least 3 samples of cyanobacteria were analyzed; (2) BMAA positive results in the free fraction of AQC with HPLC-FLD analysis were not corroborated with LC-MS/MS analysis, therefore the positive result was concluded to be due to misidentification; (3) ELISA positive results were not corroborated with LC-MS/MS analysis; therefore positive result was concluded to be a false positive.; (4) Instead of the common $\mu g.g^{-1}$ DW, BMAA concentration was presented in $\mu g.L^{-1}$.

Diatoms

We have recently shown that BMAA is not only produced by cyanobacteria (as it has previously been thought) but also by the diverse phytoplankton groups, diatoms and dinoflagellates (Cox et al., 2005; Lage et al., 2014 ; **Paper II**).

Diatoms are eukaryotic unicellular photoautotrophs. Their name is derived from the Greek *diatomos*, meaning 'cut in half ', which is a reference to their distinctive two-part cell walls made of silica, called frustule (Smetacek, 1999). Diatoms have evolutionary diverged into two classes, i.e. centric and pennate, over at least 90 million years based on the fossil record (Bowler et al., 2008). Centric and pennate diatoms can be morphologically distinguished by their circular or elongated frustule structure, respectively. Diatoms are thought to be originated from a secondary endosymbiotic event between a red algae and a heterotrophic eukaryotic host (Falkowski et al., 2004). The genomes of two diatom species have been sequenced; the centric *Thalassiosira pseudonana* and the pennate *Phaeodactylum tricornutum* (Armbrust et al., 2004; Bowler et al., 2008). The sequences provided additional support for the secondary endosymbiosis theory and have shown metabolic adaptations to the surrounding environment; e.g. characterization of the features of central carbon metabolism pathways (Bowler et al., 2010; Smith et al., 2012) Recently, knowledge about diatom nitrogen metabolism has also increased, based on the proteomic and metabolomic profiles in response to nitrogen starvation (Alipanah et al., 2015; Levitan et al., 2015).

Diatoms are the most diverse group of algae and play a key role in aquatic ecosystems; representing one-fifth of the photosynthesis on Earth (Nelson et al., 1995), which generates as much organic carbon as all terrestrial rainforests combined (Nelson et al., 1995; Field, 1998). Thus, they represent the foundation of many marine food webs and are major contributors in biogeochemical processes in aquatic environments, especially the cycling of carbon and silicon (Mann, 1999; Sarthou et al., 2005).

The supporting frustule allows the cell to grow large, which means a rather low surface to volume ratio of the plasma membrane (Chisholm, 1992). This feature makes diatoms inferior competitors in oligotrophic waters, contrary to the smaller phytoplankton, like cyanobacteria. Even so, diatoms are more efficient in utilizing nutrients and therefore stronger competitors in nutrient rich environments. Eutrophication, being it anthropogenic or natural, might therefore result in diatom, cyanobacteria and dinoflagellates blooms. It has long been known that blooms of phytoplankton can be deleterious to aquatic organisms or humans (Boesch et al., 1997). Such blooms are referred to as harmful algal blooms (Landsberg, 2002). Only a small number of diatom species, e.g. *Coscinodiscus centralis, Coscinodiscus concinnus, Coscinodiscus wailesii, Chaetoceros convolutus, Asterionellopsis glacialis, Cera-*

toneis closterium, Anaulus australis, Thalassiosira mala, and a few species of the *Pseudo-nitzschia* genus are recognized as harmful. This through production of either a toxin, exudates, mechanical damage due to cell morphology, and/or high biomass accumulation (Smetacek, 1985; Fryxell and Villac, 1999; Villac et al., 2010). However, this list of species does not account for the diatom species that produce BMAA, which so far have shown to be a common feature among all the tested diatom species (Réveillon et al., 2015; Reveillon et al., 2016 ; **Paper II** and **III**).

Marine toxins: producers, functions and effects

Algal blooms have many detrimental consequences for the aquatic ecosystems, even when the phytoplankton species do not produce toxic compounds. For instance, phytoplankton blooms can increase the turbidity of aquatic ecosystems, shading aquatic plants and thereby destroy important invertebrate and fish habitats (Paerl and Huisman, 2008). Moreover, when blooms disrupt and decay they may deplete oxygen in their surroundings, thus causing fish to die (Paerl and Huisman, 2008).

Toxin production by cyanobacteria and diatoms (Table 2) may lead to various implications in mammals depending on the target of toxicity. They can have neurotoxic characteristics causing the inhibition of neuronal control over ion concentrations across the cell membrane, which ultimately cause neurological insults in mammals; or they can show hepatotoxic characteristics, causing injuries of liver and/or by internal hemorrhages. Although their effects in humans have been well documented (Narahashi, 1972; Berman and Murray, 1997; Sivonen and Jones, 1999; Pulido, 2008; Sivonen and Börner, 2008; Etheridge, 2010), despite much speculation and development of several hypotheses, the ecological or physiological functions of most toxins produced by phytoplankton have remained largely unknown. Nevertheless, biosynthesis of these toxins requires a high input of energy, which suggests that their production needs to be advantageous for their producers (Sivonen and Börner, 2008). Nutrition availability, as well as light and temperature are often displayed as a triggering factor in the production of some well-known phytoplankton toxins (Fehling et al., 2004; Neilan et al., 2013; Van de Waal et al., 2014; Harke and Gobler, 2015).

Anatoxins, i.e. anatoxin-a, homoanatoxin-a and anatoxin-a(s), are a group of neurotoxins isolated from cyanobacteria (Devlin et al., 1977; Matsunaga et al., 1989). Anatoxin-a is potentially the most common cyanobacterial neurotoxin and is produced by *Anabaena flos-aquae, Anabaena spp., A. planktonica, Aphanizomenon spp., Cylindrospermum spp., Oscillatoria spp., Planktothrix rubescens, Phormidium flavosum, Arthrospira fusiformis* (Sivonen and Jones, 1999; Viaggiu et al., 2004; Gugger et al., 2005). The physicochemical parameters (temperature, light intensity and nitrogen source) and growth phase have been found positively correlated with the toxin production (Rapala et al., 1993; Gallon et al., 1994; Gupta et al., 2002).

However, in contrast to the other toxins, anatoxins are produced under conditions suboptimal for growth, e.g. during nitrogen starvation (Saker and Neilan, 2001). Thus, most probably production of anatoxins is not a direct function of cell growth, as suggested for the production of microcystins and nodularins (Long et al., 2001; Neilan et al., 2013).

Saxitoxins are potent neurotoxins belonging to a group of structurally related toxins known as the paralytic shellfish toxins. These toxins are produced by both eukaryotic marine dinoflagellates, i.e. *Alexandrium* species, *Pyrodinium bahamense*, and *Gymnodinium catenatum*, as well as freshwater cyanobacteria of the genera *Anabaena*, *Cylindrospermopsis*, *Aphanizomenon*, *Lyngbya*, *Raphidiopsis*, *Planktothrix* and *Scytonema* (Shumway, 1990; Neilan et al., 2013). Several studies have correlated the production of saxitoxins with the availability of macronutrients, temperature and light intensity. However, these studies have used different organisms and methods used for toxin detection; thus, the results have so far been inconclusive (Sivonen and Börner, 2008; Neilan et al., 2013).

Table 2. Summary of most commonly studied cyanobacteria and diatom toxins.

Chemical group	Toxins	Mechanism of action	Source organisms
Alkaloids	Anatoxins (neurotoxic)	Binding irreversibly to acetylcholine receptors	Cyanobacteria: *Anabaena flos-aquae, Anabaena sp., A. planktonica, Aphanizomenon sp., Cylindrospermum sp., Oscillatoria sp., Planktothrix rubescens, Phormidium flavosum, Arthrospira fusiformis*
	Saxitoxins (neurotoxic)	Binding and blocking the sodium channels in neural cells	Cyanobacteria: *Anabaena circinalis, Aphanizomenon spp., Lyngbya wollei, Cylindrospermopsis raciborskii , Raphidiopsis brookii, Raphidiopsis sp., Planktothri sp., Scytonema sp.* and Dinoflagellates: *Alexandrium sp., Pyrodinium bahamense, Gymnodinium catenatum*
	Domoic Acid (Neurotoxin)	Binding irreversibly to glutamate receptors	Diatoms: *Pseudo-nitzschia sp.*
	Cylindrospermopsin (Hepatotoxic)	Blocks protein synthesis	Cyanobacteria: *Cylindrospermopsis raciborskii*
Cyclic Peptides	Microcystins (Hepatotoxic)	Inhibition of protein serine/threonine phosphatases 1 and 2A	Cyanobacteria: *Microcystis, Oscillatoria/Planktrotrix, Anabaena, Nostoc, Anabaenopsis*
	Nodularins (Hepatotoxic)		Cyanobacteria: *Nodularia spumigena*

Domoic acid (DA) is a neurotoxin which causes persistent neurological symptoms (Wright et al., 1989) and triggers the so called amnesic shellfish disorder in humans (Bates et al., 1989). DA is produced by several strains of the diatom species *Pseudo-nitzschia* (Lelong et al., 2012). The macronutrient and micronutrient availability,

nitrogen source, growth phase, bacterial community and age of cultured isolate have been shown to influence the DA production (Bates et al., 1995; Pan et al., 2001; Fehling et al., 2005; Wells et al., 2005; Thessen et al., 2009).

Cylindrospermopsin is produced by various genera of filamentous cyanobacteria, and has hepatotoxic, nephrotoxic and general cytotoxic effects in mammals, as well as potential carcinogen qualities (Humpage et al., 2000; Runnegar et al., 2002; Froscio et al., 2003). Nitrogen source (NO_3^-, NH_4^+ or N_2), light intensity, sulfate and phosphate availability have been shown to affect the production of cylindrospermopsin (Saker and Neilan, 2001; Bácsi et al., 2006; Dyble et al., 2006).

The hepatotoxic microcystins are the largest and most structurally diverse group of cyanobacteria toxins (Welker and von Döhren, 2006) and their biosynthesis is thought to be influenced by a number of different physical and environmental parameters, including nitrogen, phosphorous, trace metals, growth temperature, light and pH (van der Westhuizen and Eloff, 1985; Sivonen, 1990; Lukač and Aegerter, 1993; Song et al., 1998). Microcystins have been identified in the planktonic species *Microcystis, Oscillatoria/Planktrotrix, Anabaena* (now *Dolichospermum*), *Nostoc* and *Anabaenopsis*, from which *Microcystis* is the most common producer (Sivonen and Jones, 1999).

Nodularin, like microcystin, is a potent hepatotoxin produced by cyanobacteria. It originates from *Nodularia spumigena* (Ohta et al., 1994). The stimulation of toxic production, both microcystin and nodularin, occurs under conditions that promote optimal growth, such as the eutrophication of aquatic ecosystems by large quantities of nitrogen and phosphorus (Mazur-Marzec et al., 2006; Jonasson et al., 2008; Neilan et al., 2013; Harke and Gobler, 2015).

With regard to BMAA in cyanobacteria, there are a few studies of the environmental factors affecting the production of BMAA or the effects of added BMAA (Downing et al., 2011; Downing et al., 2012; Berntzon et al., 2013). Nitrogen starvation was found to induce the production of free BMAA in the non-nitrogen-fixing cyanobacteria *Microcystis* and *Synechoscystis* and the free BMAA in *Microsystis* disappeared within one h when either ammonium or nitrate was added to the culture (Downing et al., 2011). However, N-starvation did not promote BMAA biosynthesis in the nitrogen-fixing *Nostoc sp.* nor in the non-nitrogen-fixing *Leptolyngbya sp.* (Reveillon et al., 2014). Added BMAA was shown to inhibit nitrogen fixation in the nitrogen-fixing *Nostoc sp*. (Berntzon et al., 2013) and loss of photopigments and decrease in cell growth, in both *Nostoc sp.* and the non-nitrogen-fixing *Synechoscystis sp.* (Downing et al., 2012; Berntzon et al., 2013). Furthermore, a study conducted in *Synechoscystis sp.*, suggests that the enzyme GOGAT, through transamination reactions, might metabolize BMAA (Downing and Downing, 2016). In relation to diatoms, **Paper IV** is the first study where the effects of added BMAA are studied.

Nitrogen cycle: diatoms and cyanobacteria - the similarities

Diatoms and cyanobacteria are phylogenetically apart; belonging to even different domains, Eurkarya and Bacteria, respectively (Raven and Giordano, 2014). In spite of this, it had been shown that diatom cells respond to nitrogen deficiency in a way more similar to the responses of cyanobacteria than to those of other eukaryotes, such as green algae and higher plants (Hockin et al. 2012). The nitrogen metabolism of these two phytoplankton groups share several characteristics (Table 3).

Table 3. A summary of assimilation and incorporation of nitrogen in diatoms and cyanobacteria.

	Diatoms	**Cyanobacteria**
Nitrogen Sources	nitrate, ammonium, urea and amino acids	nitrate, ammonium, urea, amino acids and dinitrogen
Incorporation of nitrogen	GS/GOGAT-pathway	GS/GOGAT-pathway
Form of nitrogen incorporated in the GS/GOGAT-pathway	Ammonium	Ammonium
Enzymes	NADH-nitrate reductase and Fd-nitrite reductase	Fd-nitrate reductase and Fd-nitrite reductase
Types of GS	GSII and GSIII	GSI and/or GSIII, depending on species
Types of GOGAT	Fd-GOGAT , NAD(P)H-GOGAT and NADH-GOGAT	Fd-GOGAT and NADH-GOGAT
Molecules needed from carbon metabolism	ATP and 2-oxoglutarate	ATP and 2-oxoglutarate

The nitrogen sources most commonly used by cyanobacteria are nitrate, ammonium, urea, dinitrogen and some amino acids (Herrero and Flores, 2008). Nitrate assimilation involves incorporation into the cell through an active transport system and the intracellular two-step reduction to ammonium sequentially catalyzed by the ferredoxin-nitrate reductase and ferredoxin-nitrite reductase (Frias et al., 1997; Sakamoto et al., 1999; Flores et al., 2005; Flores and Herrero, 2005). However, ammonium can also been taken up directly from the medium through permeate biological membranes or with the help of ammonium permeases (Kaneko et al., 1996; Montesinos et al., 1998). Ammonium-repressible ureases have also been described in cyanobacteria, which allow them to use urea as a nitrogen source (Flores et al., 2005).

Several cyanobacteria species are able to fix atmospheric nitrogen, under aerobic conditions. Due to the extremely oxygen sensitivity of the nitrogen fixation enzymatic complex (nitrogenase), cyanobacteria separate, either spatially or temporarily, the processes of oxygenic photosynthesis and nitrogen fixation (Fay, 1992; Haselkorn and Buikema, 1992). For instance, some filamentous cyanobacteria (e.g.

genera *Anabaena* and *Nostoc*) confine nitrogenase to heterocysts-differentiated cells specialized in nitrogen fixation (Haselkorn and Buikema, 1992). Some unicellular as well as a few filamentous strains express the nitrogenase activity only during the dark periods of the light-dark growth cycles (Ohki et al., 1992; Toepel et al., 2008). Regardless of which way ammonium enters the cyanobacterial cell, it is subsequently incorporated into carbons skeletons, mainly through the glutamine synthetase /glutamate synthase pathway (GS/COGAT) (Flores et al., 2005). Two types of GS (GSI and GSIII) and two types of GOGAT (ferredoxin-GOGAT and NADH-GOGAT) have been described in cyanobacteria (Muro-Pastor et al., 2005). Carbon skeletons required for ammonium assimilation are supplied in the form of 2-oxoglutarate, which is synthesized by isocitrate dehydrogenase (Muro-Pastor et al., 2001, 2005).

Diatoms, as many other phytoplankton groups including cyanobacteria, utilizes inorganic nitrogen in the form of ammonium or nitrate (Dham et al., 2005), and organic nitrogen like amino acids and urea (Baker et al., 2009; Solomon et al., 2010). After entering the cell, nitrate is first reduced to nitrite by the cytosolic NADH-dependent nitrate reductase (Allen et al., 2005). Nitrite is then transported into the chloroplast and further reduced to ammonium by a cyanobacterium-like ferredoxin-dependent nitrite reductase (Bowler et al., 2010). Ammonium, which can also freely enter the cell, is assimilated by the GS/GOGAT pathway to amino acids and other nitrogenous compounds (Zehr and Falkowski, 1988; Takabayashi et al., 2005). Due to its secondary endosymbiosis origin, diatoms possess a plastidial glutamine synthetase (GSII) (Siaut et al., 2007) plus the glutamate synthase (Fd-GOGAT) as well as mitochondrial NAD(P)H-GOGAT and the GSIII which also have been found in cyanobacteria (Bowler et al., 2010; Allen et al., 2011). Mitochondrial GSIII may catalyze the assimilation of glutamine from ammonium derived from cytosolic catabolic reactions, e.g. deamination and hydrolysis of organic nitrogen (Parker and Armbrust, 2005; Hockin et al., 2012). Like in all other eukaryotic microalgae, in diatoms, the intermediate metabolism of carbon and nitrogen metabolism are closely interconnected and centered on available glutamate and 2-oxoglutarate (Levitan et al., 2015). Also in common with cyanobacteria, diatoms possess a complete urea cycle (Armbrust et al., 2004), which potentiates the efficiency of nitrogen re-assimilation from catabolic processes (Allen et al., 2006).

Aims

Increasing evidence suggests a link between BMAA and neurodegeneration. Moreover, the occurrence and bioaccumulation of BMAA within terrestrial as well as aquatic ecosystems around the world have been continuously reported. However, the bioaccumulation patterns of BMAA in aquatic ecosystems have not yet been tested with sufficient sample sizes able to statistically corroborate the bioaccumulation hypothesis. In addition, in most studies where natural populations of phytoplankton are collected and analyzed, the characterization of species are not performed - which ultimately contributes to the uncertainty of the BMAA producers. Add to this, there is a lack of validated extraction protocols for BMAA, which leads to incomparable results between studies. The environmental conditions, which promote phytoplankton production of the neurotoxin BMAA, are also unknown. In order to help solving these issues, this thesis aims to:

- Study the potential BMAA production in a freshwater system, Lake Finjasjön.
- Examine the BMAA bioaccumulation pattern in a freshwater environment, using a large number of fish samples, to allow a statistical approach.
- Investigate whether, in addition to cyanobacteria, other phytoplanktonic groups are able to produce BMAA.
- Evaluate different commonly used methods for the extraction of BMAA with criteria: linearity, precision, accuracy, matrix effect and recovery.
- Establish an in-house validation of a method for the extraction of BMAA.
- Take the first steps in the analysis of effects of added BMAA to the metabolism of diatoms.

Comments on methods

Finjasjön field samples

In **Paper I**, Lake Finjasjön, located in southern Sweden (56°08' N, 13°42' E), was used as a eutrophicated model lake in order to study the BMAA bioaccumulation patterns in fish.

Finjasjön has been considered as a eutrophicated lake since the early 20th century and consequently is annually affected by major blooms of toxic cyanobacteria, mainly *Aphanizomenon klebahnii* and *Microcystis aeruginosa* (Annadotter et al., 1999; Annadotter and Forssblad, 2011). Over the years, multiple techniques have been unsuccessfully applied with the intention of restoring the natural state of Finjasjön (Annadotter et al., 1993; Annadotter et al., 1999). In 1992, a top-down control strategy was implemented, by reducing the populations of planktivorous and benthivorous fish, in this case the cyprinids *Abramis brama* (bream) and *Rutilus rutilus* (roach). This strategy managed to increase the population of zooplankton and consequently the grazing pressure by zooplankton on phytoplankton (Annadotter et al., 1993; Annadotter et al., 1999). Hence, after the biomanipulation of Finjasjön, the water transparency and the native fauna and flora composition was recovered (Annadotter et al., 1999). Finjasjön has successfully been biomanipulated. Since 1992 and up until 2007 the two cyprinid species have been intermittently removed by trawling. From 2010 until this date the cyprinids have been removed annually, by fyke netting during the spring spawning and in the fall by ring seining (Annadotter and Forssblad, 2011; Annadotter and Sheet, 2014). An important fact during our study is that the pelagic - piscivorous and plankti-benthivorous - fish species were found to exist in equal proportion during spring 2012 (Annadotter and Sheet, 2012).

203 fish individuals were caught throughout two seasons, i.e. fall (September and October) 2011 and spring (April) 2012, and water samples were collected from the upper surface water in April 2012. The selection of fish species was based on the trophic level and habitat. The number of individuals per species and the proportional of females/males were random, depending on the catch (Table 4). In addition, the fish species *Tinca tinca* (tench) n = 15, *Lota lota* (burbot) n = 6, *Salmo trutta trutta* (trout) n = 6, *Gymnocephalus cernua* (ruffe) n = 15, *Scardinius erythrophthalmus* (common rudd) n = 10, and *Anguilla Anguilla* (eel) n = 15 were caught only in

spring. Only data from fish species collected during both seasons were used for statistical analysis in order to have an analogous sample size between seasons. Weight and gender for all fish samples were determined prior to dissecting of brain, muscle, liver, and kidney. Samples were later analysed for BMAA content.

Table 4. Number of fish individuals (females and males) collected in both seasons.

	Fall 2011		**Spring 2012**	
Species	**Female**	**Male**	**Female**	**Male**
Abramis brama (bream)	0	7	14	11
Perca fluviatilis (perch)	7	2	20	0
Esox lucius (pike)	1	6	9	6
Sander lucioperca (pike-perch)	1	10	16	2
Rutilus rutilus (roach)	5	4	15	0

Phytoplankton cultures and field samples

In **Paper II**, the potential production of BMAA by diatoms was investigated. Laboratory diatom cultures were selected based on the species natural occurrence in the Baltic Sea and Swedish West Coast (Table 5). Additionally, both pelagic and benthic field samples were collected near Kristineberg Marine Research Station, on the Swedish West Coast (58.2°N, 11.3°E) in summer 2010. The dominant cyanobacterial and diatom morphotypes in the individual samples were determined by microscopy analysis using an Olympus BH-2 microscope equipped with a digital camera.

Table 5. Marine diatom strains, from **Paper II**.

Species	**Strain number**	**Collection place**
Achnanthes sp.	[1]CCAP 1095/1	Millport, Scotland
Navicula pelliculosa	[1]CCAP 1050/9	Massachusetts, USA
Proboscia inermis	[1]CCAP 1064/1	Brandsfield Strait, 63°15´S 58°20´W
Skeletonema marinoi	[2]SAAE08603	Gullmarsfjorden, Sweden
Skeletonema marinoi	[2]ST28	Strömstad, Sweden
Thalassiosira sp.	[1]CCAP 1085/15	Loch Linnhe, UK 56°28´N 50°30´W

[1]Culture Collection of Algae and Protozoa, Scottish Marine Institute, Dunbeg, Oban, Scotland, [2]Provided by Prof. Anna Godhe, Department of Biological and Environmental Sciences, University of Gothenburg, Sweden.

One of the Kristineberg field samples, containing a mixture of the cyanobacteria *Leptolyngbya sp.* and the diatom *Naviculales*, was treated with germanium dioxide for three weeks in order to eliminate the diatoms. The diatoms absorb the germani-

um instead of silica, which results in their frustule disintegration and ultimately the death of the diatom, while cyanobacteria remain unaffected (Andersen, 2005). Thus, this experiment allowed us to test if the diatoms alone contain BMAA, in the field sample.

BMAA exposure experiment

In **Paper IV**, two BMAA producing diatom species, *Phaeodactylum tricornutum* SCCAP K-1280 and *Thalassiosira weissflogii* GUMACC123 (**Paper III**), were exposed to exogenous BMAA. Diatoms were shown to produce higher BMAA concentrations than cyanobacteria (**Paper III**; Reveillon et al., 2015; Reveillon et al., 2016). Nevertheless, in **Paper IV** diatoms were exposed from 2 to 20000 times lower BMAA concentrations than cyanobacteria have been previously exposed (Downing et al., 2012; Berntzon et al., 2013; Downing and Downing, 2016). Thus, in **Paper IV** the concentrations of exogenously applied BMAA mimic the BMAA concentrations that phytoplankton are naturally exposed to. Consequently, the obtained results of the physiological effects of BMAA exposure in diatoms will not be obscured by atypical concentrations. The effects of exposure were evaluated by the analysis of chlorophyll *a*, protein and BMAA concentration in the diatoms and by measurements of the extracellular nitrite and ammonia concentrations.

BMAA extraction protocols and validation

In **Paper III,** the issue of BMAA extraction protocols as a source of quantification inconsistencies between separate studies is addressed. Therefore, the focus of **Paper III** is to evaluate the efficiency of three BMAA extraction methods, commonly used in BMAA research, in both BMAA-spiked cyanobacteria (i.e. *Spirulina* powder) and in one non-spiked diatom culture. Thus, an in-house validation, taking into account the methods linearity, precision (i.e. repeatability and intermediate precision), accuracy, matrix effect and recovery, was performed. Method A (used in **Paper I**) was the first method used to extract BMAA from aquatic organisms (Jonasson et al., 2010; Spacil et al., 2010). This method comprises BMAA extraction in 80% methanol (MeOH) and protein hydrolysis in 6 M hydrochloric acid (HCl) for 20 h at 110 °C, followed by a solid-phase-extraction (SPE) clean-up. Relatively high BMAA losses were registered in the recovery experiment of **Paper I**, where the SPE step was suspected to be the cause. Thus, the efficiency of Method A without the SPE step was tested in **Paper III**. Method B is the first method applied to extract BMAA from diatoms and based on a 1:2, 20% MeOH:acetone protein precipitation followed by protein hydrolysis (see **Paper II**). In Method B, the BMAA is fractionated into free and protein-associated forms. This fractionation is also done in Method C; however, in Method A the BMAA results represent the total BMAA amount in the sample. Method C, used in in **Paper IV,** is the most commonly used protocol in BMAA research and is based on a trichloroacetic acid (TCA) protein precipitation (Murch et

al., 2004a; Faassen, 2014). It also comprises a protein hydrolysis step and a clean-up step with a centrifugal filter unit. Since the AQC derivatization is a shared step among these three protocols, the derivatization effectiveness was also tested in **Paper III.** Protein hydrolysis is also a joint step among all methods (i.e. method A, B and C) that analyze protein bound BMAA (Murch et al., 2004a; Spacil et al., 2010 ; **Paper II**). Hydrolysis with HCl is the most commonly used method of releasing amino acids from proteins, including BMAA (Fountoulakis and Lahm, 1998; Banack et al., 2007; Cohen, 2012). Published methods for analyzing bound BMAA usually employ 6 M HCl hydrolysis at 110 °C *in vacuo*; however, the times vary from 12 to 24 h , conditions that are sufficient to release BMAA quantitatively (Banack et al., 2007; Cohen, 2012).

UPLC-ESI-MS/MS

Liquid chromatography-mass spectrometry combines the versatility of LC, a technique used to separate a wide range of compounds (such as macromolecules, ionic species, polar and high-molecular weight compounds) in a liquid mobile phase (Dong, 2006) with the sensitivity of MS (Niessen, 2006). Thus, LC-MS is a highly sensitive and selective method for identification of natural products in complex mixtures (Fredenhagen et al., 2005; Furtado et al., 2007). Compounds are first separated in the column of the LC system and later directed to the mass spectrometer by a flow separator. They are then ionized in the flow separator and further separated in the mass analyzer according to their (*m/z*) ratio (Fig. 6) (Niessen, 2006).

In this thesis, i.e. **Paper I, II, III** and **IV**, the instrument ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC-ESI-MS/MS) was employed and therefore, electrospray ionization (ESI) is used. ESI uses electrical energy to assist the transfer of ions from solution into the gaseous phase before they are subjected to MS analysis. This involves three consecutive steps; dispersal of a fine spray of charge droplets, solvent evaporation and ion ejection from the highly charged droplets tube, which is maintained at a high voltage relative to the wall of the surrounding chamber (Ho et al., 2003). Tandem mass spectrometer consisting of two quadrupole mass analyzers in series, i.e. Q_1 and Q_3, with a non-mass-resolving quadrupole between them to act as a cell for collision-induced dissociation, i.e. Q_2 (Pitt, 2009). In this analyzer, an ion (called precursor ion) from the first stage of MS is selected and activated, to produce fragment ions, which are then analyzed in the second stage of MS. In other words, a precursor ion is selected by mass-to-charge ratio (*m/z*) on the first quadrupole (Q_1), fragmented by collision-induced dissociation in Q_2, and fragment ions are detected by selected *m/z* in Q_3 (Pitt, 2009). In the end, the detected signal (fragment peak) is proportional to the amount of the analyte in the sample (Fig. 6).

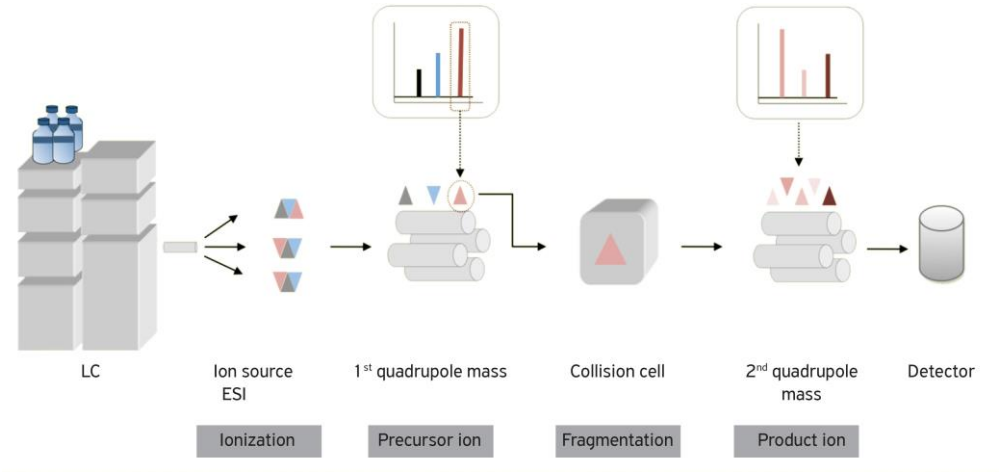

Figure 6. Scheme of the basic components of UPLC-ESI-MS/MS, adapted from Torre et al. (2015). Q_1 is labeled as 1st quadrupole mass, Q_2 as collision cell and Q_3 as 2nd quadrupole mass.

The proven superiority of UPLC-ESI-MS/MS over other methods has made it the method of selection in BMAA research (Cohen, 2012; Faassen et al., 2012; Faassen, 2014). The main reason for using UPLC-ESI-MS/MS is due to the BMAA structural isomers. These isomers have been one of the major sources of interferences on analytical methods, due to their analogous chromatographic retention properties and identical mass. Issues that are overtaken with the UPLC-ESI-MS/MS, since precise values of retention time, *m/z* of the precursor ion, pattern of the fragmentation spectrum after collision induced dissociation and the ratio between the intensities of specific ions MRM transitions, needs to be reached in order to positively identify BMAA (Faassen et al., 2012; Jiang et al., 2012). Detection of BMAA in the biological samples was based on the quantifier fragment 459.1>119.08, also common to the BMAA isomers, and the qualifier fragment 459.1>258.09. For the isomers N-(2-aminoethyl) glycine (AEG) and 4-diaminobutyric acid (DAB) the qualifier fragments were 459.1>214.1 and 459.1>188.1, respectively. Retention time and ratio of fragments 119.08/258.09 was continuously controlled by comparing with the BMAA standard in the corresponding matrix (see **Paper III**).

In **Paper I, II, III** and **IV**, an AQC-Tag Ultra Derivitization Kit (AQC) is used prior to UPLC-ESI-MS/MS analysis. This method has been described as fast, reproducible and a sensitive amino acid quantitation method for biological samples, yielding a ~100% amino acid conversion (Armenta et al., 2010). AQC has been shown to improve limits of detection (LOD) up to 3 times while reducing the analysis time 2.5 fold (Boogers et al., 2008). Derivatization is required to increase the mass of the target amino acid, improving the Signal/Noise (*S/N*) ratio, doubly labelling it, which makes the amino acid more hydrophobic, and improves the ESI response by increasing the analyte surface activity. However, in BMAA research other derivatization agents, e.g. propyl chloroformate (EZ:faast, Phenomenex, Torrance, USA); 9-

fluorenylmethyl chloroformate and dansyl chloride, have been used instead of AQC (Esterhuizen and Downing, 2008; Scott et al., 2009; Esterhuizen et al., 2011; Salomonsson et al., 2013). In addition, methods without derivatization steps, relying exclusively on MS analysis with separation using hydrophilic interaction chromatography have also been used in the detection of BMAA (Rosén and Hellenäs, 2008; Faassen et al., 2009; Kruger et al., 2010; Faassen et al., 2012). One of the disadvantages of AQC is that it reacts with both primary and secondary amino groups, i.e. reacts with all amino acids and other compounds containing amino groups (Cohen and Michaud, 1993; Kaspar et al., 2009). Thus, in complex biological samples this may hamper the derivatization efficiency, accurate separation and ultimately the analysis of BMAA (Rosén and Hellenäs, 2008; Eriksson et al., 2009; Faassen et al., 2012). Another disadvantage is that as a diamino acid, BMAA may react more slowly compared to other amino acids. In addition, it might also be single or double derivatized (Cohen, 2012). Quantification of single derivatized BMAA would be less prone to interferences by other components present; however most published studies quantify double-derivatized BMAA (Cohen, 2012). These disadvantages can be overtaken if derivatization completion is assured through an adequate ratio of total protein to derivative (i.e. the AQC reagent present in excess) (Eriksson et al., 2009; Cohen, 2012 ; **Paper III**).

Statistical analysis

Parametric linear models were applied to the data collected in **Paper I**, in order to detect any significant ($p < 0.05$) influence of the variables season of collection, fish gender, total weight, and species on the response variable (i.e. BMAA concentration in fish brain tissue) distribution. Supplementary Anova-Chi-Square tests were performed to confirm linear model results. The data from BMAA concentration in fish muscle tissue did not satisfy the parametric test assumptions, even after data transformation, therefore a non-parametric Spearman's rank correlation coefficient was used to test whether the BMAA concentrations in fish muscle tissue were correlated ($p < 0.05$) with the brain tissue. In **Paper IV**, analysis of variance between the concentrations of chlorophyll a, total protein, BMAA (both free and protein-associated), ammonia and nitrite of a treated sample harvested at different time points and of treated sample and controls harvested at the same time points was achieved with one-way ANOVA. Two-way ANOVA analysis was performed in order to determine interactions of the measured data with treatment and time. Statistical analysis of **Paper I** and **IV** was carried out on R Statistical Software (Foundation for Statistical Computing, Vienna, Austria). Statistics applied in **Paper II, III** were executed on Microsoft Office Excel 2010 (Microsoft Corporation, WA, USA).

Results and discussion

BMAA bioaccumulation in aquatic ecosystems

The biomagnification of BMAA in an aquatic ecosystem was first reported in the Baltic Sea, where the BMAA biosynthesis was suggested to be most likely done by the cyanobacteria species *Nodularia* and *Aphanizomenon*. The BMAA produced by the cyanobacteria was possibly transferred to marine animals that directly or indirectly feed on them, i.e. zooplankton, fish, mussels and oysters, with highest BMAA concentrations in bottom-feeding fish species as well as filter feeding mollusks (Jonasson et al., 2010). The same trend of bioaccumulation was later found in South Florida coastal waters in USA and in Lake Taihu in China (Brand et al., 2010; Mondo et al., 2012; Jiao et al., 2014). At this point, the theory of BMAA bioaccumulation in aquatic ecosystem had solid proofs; however, none of the studies used enough replicates to allow a statistical approach, which was the main objective of **Paper I**.

In Finjasjön, BMAA was detected in water samples (0.002 ± 0.001 to 0.006 ± 0.002 $\mu g.g^{-1}DW$) at comparable concentrations as in the Baltic Sea cyanobacteria (Table 1) (Jonasson et al., 2010). However since Finjasjön suffer from both cyanobacteria and diatom blooms year-round (Annadotter and Forssblad, 2011), the source of BMAA is questionable (see **Paper II**).

In **Paper I**, the incidence of BMAA was higher in the plankti-benthivorous fish, i.e. *Abramis brama* (bream) and *Rutilus rutilus* (roach), compared to the pelagic - piscivorous fish species, i.e. *Esox lucius* (pike) and *Sander lucioperca* (pike-perch) (Fig.7). This is in accordance with previous studies, where BMAA accumulation preferably was found in the benthic systems (Brand et al., 2010; Jonasson et al., 2010; Mondo et al., 2012; Jiao et al., 2014). Thus, the results presented in **Paper I** re-affirm the hypothesis of phytoplankton descending in the water column during the bloom decline, making BMAA available for the bottom feeders, and/or that benthic phytoplankton contain higher concentrations of BMAA than the pelagic species.

The continuous removal of *A. brama* and *R. rutilus* from Finjasjön, through the food web manipulation scheme, resulted in a diet change, towards an increase in feeding on benthic resources by the remaining population of this species (Persson and

Hansson, 1999). Between the two plankti-benthivorous species, higher concentrations of BMAA were found in *A. brama*, which can be explained by the more restricted benthic feeding by this species in comparison to *R. rutilus*, which occasionally moves to the open-water in order to feed (Persson and Hansson, 1999).

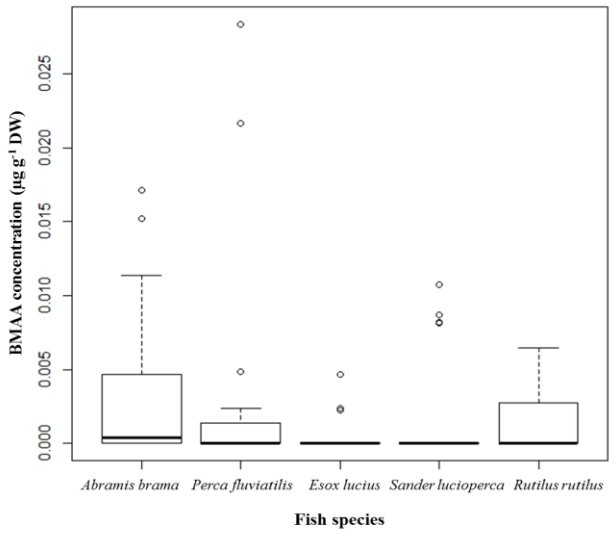

Figure 7. Total BMAA concentrations (µg.g^{-1}DW) in brain tissue of fish species from Lake Finjasjön; *Abramis brama*, *n* = 32; *Perca fluviatilis*, *n* = 29; *Esox Lucius*, *n* = 22; *Sander lucioperca*, *n* = 29; and *Rutilus rutilus*, *n* = 24; median, 75th quartile, maximum, and outliers, from **Paper I**.

Although BMAA was more broadly found in plankti-benthivorous fish, the pelagic - piscivorous fish contained some of the highest concentrations of BMAA in their brain tissue, endorsing the hypothesis of BMAA biomagnification in the Finjasjön trophic chain (see **Paper I**). The presence of BMAA in *S. lucioperca* collected in Finjasjön, but not in the same species collected in the Baltic Sea (Jonasson et al., 2010) may be explained by the small dimensions of the lake, where the susceptibly of feeding of BMAA-contaminated prey is higher in comparison to the Baltic Sea.

Some of the highest concentrations of BMAA quantified in **Paper I** was in brain samples of *Perca fluviatilis* (perch). In Finjasjön, small individuals (<80 mm) of this fish species consume 78% of the zooplankton and 22% of the benthic prey, while the larger individuals (>180 mm) feed almost exclusively on fish (Persson and Hansson, 1999). It can be hypothesized that the higher BMAA concentrations are a cumulative effect based on early zooplankton and benthic feeding followed by a subsequent constrained feeding pattern on BMAA-contaminated prey fish.

The statistical analysis corroborated the hypotheses that the fish species and the total weight of the individuals are significant in relation to the BMAA concentrations in

the brain tissues. Therefore, a fish species that has a more uniform BMAA exposure as they grow, i.e. continuous benthic feeding, will also have a higher proportion of individuals with accumulated BMAA. Besides, in a system in which BMAA is regularly available, a fish individual will most probably bioaccumulate BMAA throughout its life, so heavier individuals, likely older, will contain higher concentrations of BMAA (**Paper I**). No statistically significant relations were found between the concentrations of BMAA and the variables gender, and season of collection. However, some tendencies were observed e.g. higher concentrations of BMAA were quantified in females compared to males and individuals caught during spring as compared to the fall. Females are often larger than males (Cowx, 1983; Roff, 1983; Parker, 1992), thus, they may consume larger amounts of BMAA-contaminated prey. Most of the individuals caught in spring were females, which may explain why higher concentrations of BMAA were yielded during this season catchment.

As reported in previous studies (Brand et al., 2010; Jonasson et al., 2010; Mondo et al., 2012; Jiao et al., 2014), and in **Paper I,** the accumulation of BMAA in the different fish tissues varied. In **Paper I**, BMAA was mostly found in the fish's brain tissue, sporadically detected in the muscle tissue and neither detected in liver nor in the kidney tissue. Although not statistically tested, due to low number of positive samples, the percentage of individuals containing BMAA in the muscle tissue were higher in the plankti-benthivorous fish *A. brama* than in the other species. This might be related to the almost exclusively benthivorous diet within this species. Besides BMAA, mercury was also investigated and detected in the muscle tissue of *A. brama* and *S. lucioperca*. Although the mercury concentrations were below the EU-permitted level of 0.5mg.kg^{-1} WW (221/2002/EC), the synergetic neurotoxicity of BMAA and mercury (Rush et al., 2012) may constitute a risk for human health.

There was no correlation between the concentrations of BMAA in muscle and in brain, which may be explained by the low number of positive samples for the muscle tissue.

With regard to the fish species exclusively caught during spring, BMAA could only be detected in *G. cernua*, *T. tinca* and *A. anguilla*. In the first two species, BMAA was quantifiable in both brain and muscle tissues, which agreed with their benthic feeding (Bergman, 1991; Bergman and Greenberg, 1994). Furthermore, in *A. anguilla*, another bottom-dwelling fish species that instead feeds on the whole aquatic fauna (Deelder, 1984), BMAA could only be detected in the brain tissue.

In general, BMAA concentrations detected in the fish tissues in **Paper I** (Fig.7) are in agreement with the ones reported in the Baltic Sea study (Jonasson et al., 2010). However, the concentrations of BMAA found in fish from Finjasjön are lower than the concentrations stated in the study from Florida (58 ± 41 µg.g^{-1}WW in muscle, 588 ± 81 µg.g^{-1}WW in liver, and 1450 ± 687 µg.g^{-1}WW in kidney) (Mondo et al.,

2012); and in Lake Taihu, (concentration of BMAA in the muscle tissue was between 0.07 ± 0.021 and 35.91 ± 13.40 µg.g^{-1}DW) (Jiao et al., 2014). This discrepancy is likely due to different methods used for extraction and quantification rather than biological causes. **Paper I** and the study performed in the Baltic Sea followed the same extraction method as well as analytical method (Table 1), whereas the other studies did not (further discussed in **Paper III**).

The novel sources of BMAA – diatoms

In the study by Jonasson et al. (2010) high concentrations of BMAA were unexpectedly found in mussels harvested at the Swedish West Coast. This is an area where blooms of cyanobacteria, the known BMAA producers, are less pronounced compared to the typical bloom-forming areas in the Baltic Proper (Boesch et al., 2006). At the time, it was suggested that benthic cyanobacteria might produce higher amounts of BMAA than the bloom forming cyanobacteria, thus causing the high BMAA concentrations detected in the mussels (Jonasson et al., 2010). However, in **Paper II**, no BMAA was detected in the benthic cyanobacteria field samples from the Swedish West Coast, containing the benthic species *Calothrix sp*. and *Rivularia sp*. Instead, BMAA production was for the first time detected in a phytoplantonic organism outside the cyanobacteria phylum, i.e. diatoms. The investigation of BMAA production by this eukaryotic group was instigated since diatoms together with dinoflagellates dominate the marine environment at the Swedish West Coast (Granéli et al., 1989). BMAA production by the dinoflagellate *Gymnodinium catenatum* has also been confirmed (Lage et al., 2014).

In **Paper II,** BMAA was detected in all six diatom cultures tested, i.e. *Achnanthes* sp., *Navicula pelliculosa*, *Proboscia inermis*, *Thalassiosira sp*. and two strains of *Skeletonema marinoi*. BMAA quantification performed in the two strains of *Skeletonema marinoi* and in *Thalassiosira* sp. showed protein-associated BMAA concentrations in a range from 0.0011 ± 0.0004 and 0.00328 ± 0.0025 µg.g^{-1}DW. Additionally, unlike cyanobacteria cultures, the cultures of diatoms were shown to produce BMAA in successive generations.

To ensure that BMAA was not only produced by diatoms in culture, field samples from the Swedish West Coast with mixed-species containing both cyanobacteria and diatoms, were tested as well. Protein associated BMAA detected in the field sample comprising the cyanobacteria *Leptolyngbya* sp. and diatoms belonging to the genus *Navicula* was 0.0276 ± 0.011 µg.g^{-1}DW. However, laboratory grown cultures of *Leptolyngbya* PCC73110 has been reported to contain 0.73 µg.g^{-1}DW BMAA (Jiang et al., 2013), thus there was the possibility that the BMAA detected in the mixed field sample might be due to the cyanobacterial BMAA production. A treatment with germanium dioxide of the mixed-species sample eliminated the diatoms, while leaving the cyanobacteria intact. This allowed us to compare the BMAA concentra-

tions with and without diatoms, i.e. quantify the BMAA produced by the natural population of the cyanobacteria *Leptolyngbya* sp. The result was 0.0047 ± 0.0017 $\mu g.g^{-1}DW$ – 3-fold lower than in the non-treated sample (**Paper II**). Consequently, the BMAA production in this field sample was mainly due to the diatoms. The results in **Paper II** suggest that production of BMAA may be common among diatom species and they might produce higher concentrations of BMAA than cyanobacteria.

Table 6. BMAA concentration ($\mu g.g^{-1}$ DW) in laboratory-grown diatom and cyanobacteria cultures, from **Paper III**.

Species	**BMAA concentration $\mu g.g^{-1}$ DW**	
	Protein fraction	**Free fraction**
Navicula pelliculosa CCAP 1050/9	1.7687 ± 0.2605	0.0910 ± 0.0570
Chaetoceros socialis SCCAP K-0550	0.6187 ± 0.4362	0.1521 ± 0.0962
Coscinodiscus granii SCCAP K-1831	0.9129 ± 0.2123	0.4412 ± 0.2805
Thalassiosira weissflogii SAG 2135[A]	2.1862 ± 0.0186	0.2126 ± 0.1335
Phaeodactylum tricornutum SCCAP K-1280	0.1720 ± 0.0911	0.0097 ± 0.0082
Skeletonema marinoi SCCAP K-0669	2.5797 ± 0.1394	0.7025 ± 0.0252
Ditylum brightwellii strain 1[A]	0.0352 ± 0.0032	ND
Ditylum brightwellii strain 2[A]	0.0187 ± 0.0047	ND
Ditylum brightwellii strain 3[B]	ND	ND
Ditylum brightwellii strain 4[B]	0.0080 ± 0.0055	ND
Synechocystis PCC 6803	0.0424 ± 0.0337	ND
Anabaena PCC 7120	0.0555 ± 0.0096	ND
Leptolyngbya PCC 73110	0.0491 ± 0.0012	ND

ND: not detected; * Mean BMAA concentration ± SD; *n* = 3 technical replicates. [A] Isolated from the Baltic Sea. [B] Isolated from the North Sea.

Further, in **Paper III,** BMAA was detected in all diatom cultures tested, where five out of the seven diatom species tested were so far not known to produce BMAA (Table 6). In general, diatom cultures contained higher concentrations of BMAA compared to the cyanobacteria cultures (Table 6). Higher concentrations of BMAA in diatom cultures compared to cyanobacteria has been confirmed by other studies (Réveillon et al., 2015; Reveillon et al., 2016). The inconsistent reports with BMAA continuously detected in field samples but not in axenic cultures of cyanobacteria (Table 1) could be explained by the fact that besides by cyanobacteria, BMAA can be produced by diatoms and dinoflagellates (Lage et al., 2014 ; **Paper II** and **III**), mostly because this phytoplanktonic groups often, co-occur in the aquatic systems. Therefore, species characterization of field samples is crucial in order to identify the

BMAA producers in a certain habitat. In addition, in **Paper III**, most of quantified BMAA is present in the protein-associated form in both cyanobacteria and diatom cultures.

BMAA extraction controversy – method validation

As previously mentioned, the varying methods is often suggested to be the main source for inconsistencies between BMAA concentration found in different studies (Faassen, 2014), even when the selective LC-MS/MS system has been used (Faassen et al., 2009; Jonasson et al., 2010; Kruger et al., 2010; Faassen et al., 2012; Salomonsson et al., 2013; Al-Sammak et al., 2014; Jiao et al., 2014; Reveillon et al., 2014; Réveillon et al., 2015; Reveillon et al., 2016 ; **Paper I** and **II**). Therefore, in **Paper III,** three of the most commonly applied BMAA extraction methods were compared through an in-house validation experiment.

Method A, is based on an 80% MeOH extraction couple with a cation-exchange solid-phase extraction (SPE) procedure (Jonasson et al., 2010; Spacil et al., 2010), and was the method used in **Paper I**. In **Paper I**, a recovery experiment with D_3-BMAA, reveal a relatively high D_3-BMAA loss in brain and muscle fish tissues during the SPE procedure. Prior to the SPE step, D_3-BMAA was added to the fish blanks and fish samples. The recovery percentages of D_3-BMAA in the blank samples was 54.9 ± 2.0% for the brain and 14.3 ± 3.5% for the muscle tissues and for the fish samples the recoveries were 34.3 ± 6.2% for the brain and 10.7 ± 7.5% for the muscle tissues. As a result, the SPE step was suggested as the major cause of the low D_3-BMAA recoveries. Considering this, an adaptation of Method A, which consisted in eliminating the SPE step from the protocol, was also evaluated in **Paper III**. The other methods investigated **in Paper III** were the 1:2, 20% MeOH: acetone protein precipitation (Method B) used in **Paper II**, and the most commonly used protocol in BMAA research, the TCA protein precipitation method (Method C) (Murch et al., 2004a; Faassen, 2014), which were used in the study described in **Paper IV**.

In the linearity test, both protein precipitation methods (B and C) display good linear fit, i.e. r^2 values of 0.99 in both protein-associated as well as the free BMAA fraction (**Paper III**). Method A, with the SPE step, produced sufficient, though not ideal, linearity, r^2 of 0.97. This linearity was generated after the removal of the highest BMAA concentration point (100 $ng.mL^{-1}$) which skewed the standard curve. However, Method A, without the SPE step, did not show any linearity, r^2 of 0.74. Lack of linearity will affect all other validation parameters, and is therefore a reason to reject this extraction protocol. Thus, the accuracy and precision was not further investigated for this method.

Positive identification of BMAA in a sample corresponded to signal-to-noise ratio (*S/N*) higher than 4.0 in both BMAA product ions chromatograms, i.e. *m/z* 459.1>119.08 and *m/z* 459.1>258.09, a retention time of 2.85±0.12 min and a SRM ratio of 5.5±1.0 between the ions transitions *m/z* 459.1>119.08 and *m/z* 459.1>258.09. 119.08. The limit of detection (LOD) was established when the *S/N* was higher than 4.0 for the diagnostic product ion of BMAA (i.e. *m/z* 459.1>258.09). The area of the general BMAA product ion (i.e. *m/z* 459.1>119.08) peak is normally 3 to 10 times higher than the peak at *m/z* 459.1>258.09 (i.e. *S/N* between 10 and 30), therefore the limit of quantification (LOQ) was described as equal to the LOD, which was 0.8 ng· mL^{-1} of BMAA. The LOQ presented in **Paper III** was amongst the lowest published values (Faassen et al., 2012; Jiang et al., 2013; Combes et al., 2014), which suggests high sensitivity.

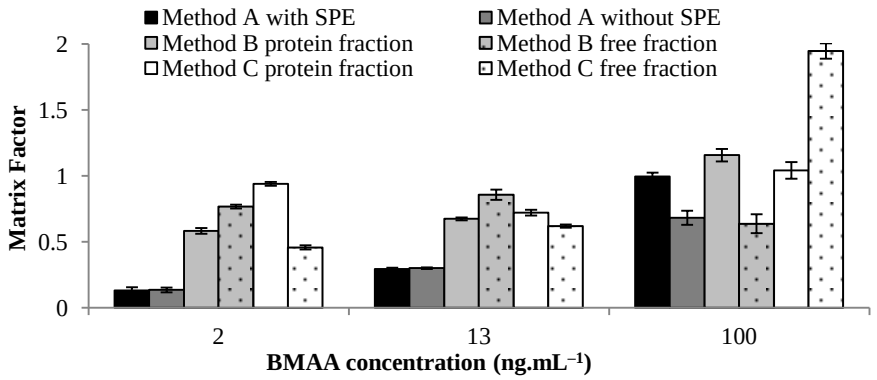

Figure 8. BMAA matrix factor ± SD in spiked cyanobacteria matrix (i.e. *Spirulina* powder, *Arthrospira fusiformis*) extracted using methods A (80% MeOH with and without SPE extraction), B (20% MeOH and acetone protein-precipitation extraction), and C (TCA protein-precipitation extraction) with 2, 13 and 100 ng.mL^{-1} quality control samples; n = 3 technical replicates, from **Paper III**.

Matrix effect, i.e. suppression or enhancement of analyte ionization by the presence of matrix components in the biological samples, as observed in Fig. 8, mainly affect the detection of BMAA in the range of lower BMAA concentrations. In **Paper III,** the signal loss is also dependent on the presence of competing amine-containing compounds, which consequently may lead to inefficient AQC derivatization (Eriksson et al., 2009; Cohen, 2012). Method A (with or without SPE) displayed a high ionization suppression, which may influence the method performance in terms of reproducibility, linearity and accuracy of a method - thus the matrix effect may explain the inability of this method to reach the validation requirements (Trufelli et al., 2011). Both Method B and C showed weak matrix interferences. Foremost-published matrix effects measured in water, biofilm and cyanobacteria matrices

(Combes et al., 2014) indicate relatively strong effects compared with the results using cyanobacterial *Spirulina* powder (Fig. 8).

As previous observed in **Paper I**, method A has very low BMAA recoveries, which is improved when samples are extracted with method A without SPE (Fig. 9), as suggested in **Paper I**. This is also consistent with a previous study that excluded the SPE step from method A and obtained recoveries of 83.5% in mussel matrix (Salomonsson et al., 2013). However, method A without SPE shows good recovery rates, but the high matrix interference, which was also observed when SPE was used (Fig. 8), and the lack of linearity, led to rejection of this method as an accurate extraction procedure. Both protein precipitation protocols (Method B and C) show good recoveries, with method C as the best (Fig. 9).

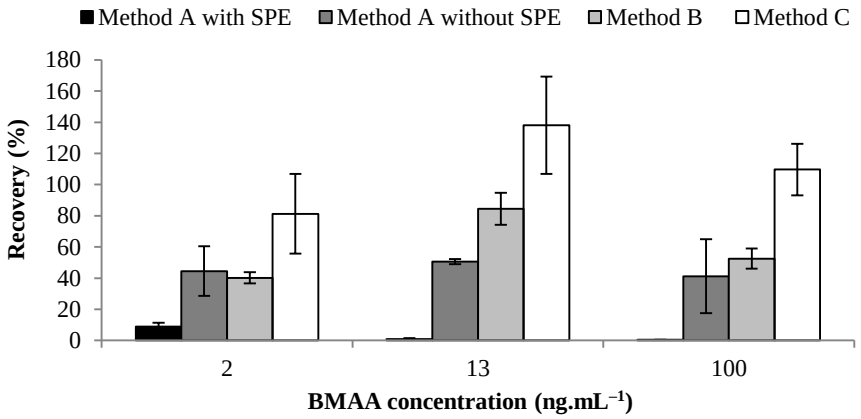

Figure 9. Recovery rates (%) ± SD in spiked cyanobacteria matrix (i.e. *Spirulina* powder, *Arthrospira fusiformis*) extracted using methods A (80% MeOH with and without SPE extraction), B (20% MeOH and acetone protein precipitation extraction) and C (TCA protein precipitation extraction) with 2, 13 and 100 ng.mL^{-1} quality control samples; *n*=3 technical replicates, from **Paper III**.

Overall, the BMAA precision, i.e. the closeness of individual analyte measurements, was under the 20 % limit established by the European Commission (98/83/EC). This 20% limit was only slightly exceeded in one of the samples from the protein fraction extracted with method C (**Paper III**). Therefore, all methods tested (method A with SPE, method B and C) show good reproducibility. Similar good precision has previously been observed in cyanobacteria matrix extracted using Method C (Combes et al., 2014; Jiao et al., 2014).

The closeness of sample mean to the true value of the analyte, i.e. the accuracy, is given as % of relative error and should range from 70 to 120 % (98/83/EC). This validation criterion was satisfied by all three methods, except for the lowest BMAA concentration (i.e. 2 ng·mL^{-1}) in the free fraction of method B (**Paper III**).

In summary, results obtained in **Paper III** where samples of cyanobacteria matrix were extracted with the TCA protein precipitation method followed by AQC derivatization and LC-MS/MS analysis (Method C) obeyed the criteria for in-house validation of methods for analyzing cyanotoxins in environmental samples according to ISO 17025 for laboratory accreditation (Kaloudis et al., 2015). Although, the other protein precipitation method (Method B) followed most of the validation criteria, the low BMAA recoveries (Fig. 9) did not permit this method to be validated. Further analysis revealed that the first pellet of method B, previously suggested to be discarded (**Paper II**), contains relatively high protein concentration, and consequently protein associated BMAA. This explains the lower BMAA concentrations found in **Paper II** compared to the BMAA concentrations detected in **Paper III** (Table 6), where the same diatom species and/or strain was tested.

The analysis of the extracted total protein by all methods also demonstrates the better performance by Method C. Method C extract four times more protein than method A and as much as both pellets combined in method B (**Paper III**). This is particularly important in BMAA analysis, because in order to achieve a complete AQC derivatization of the BMAA molecule (Cohen, 2012; Faassen et al., 2012; Faassen, 2014), a particular protein-to-derivative ratio (i.e. providing derivative in excess) needs to be complied. Thus, an accurate quantification of BMAA relies on the total amounts of protein extracted. It has previously been suggested that protein concentration should not exceed 0.6, 0.17 and 0.25 $\mu g \cdot \mu l^{-1}$ (Waters, 1993; Eriksson et al., 2009; Cohen, 2012), respectively. In **Paper III,** the optimal results were obtained when protein concentrations ranged between 0.3 and 2.3 $\mu g \cdot \mu l^{-1}$. These results were achieved through serial dilutions of a BMAA natural containing sample, i.e. laboratory culture of the diatom *S. marinoi* SCCAP K-0669.

The TCA protein extraction protocol (Method C) validated in **Paper III**, have proven to be the best method to extract BMAA from cyanobacteria matrix, and was therefore the method selected for the following study (**Paper IV**). However, one of the drawbacks of Method C is that the protein precipitation step requires a long incubation time, i.e. for 48 h. Moreover, the use of 0.1 M TCA has earlier been criticized and suggested to be too low for quantitative precipitation (Cohen, 2012). Hence, the potential of reducing the incubation time by increasing the TCA concentration was also tested in **Paper III**. Protein concentrations were, actually higher when the samples were incubated with 0.2 M TCA for 24 h, however all other concentrations tested, i.e. 0.1, 0.5, 1.0, 1.5 M TCA for 24 h were less effective than the established 0.1 $mol \cdot L^{-1}$ for 48 h (Murch et al., 2004a). In contrast, the BMAA peak areas of the product transition ion (i.e. m/z 459.1> 119.08) and the diagnostic transition ion (i.e. m/z 459.1>258.09) were much lower in any of the samples precipitated for just 24 h, leading to the conclusion that Method C does not require adjustments of the TCA concentration nor the precipitation length (**Paper III**).

Physiological role of BMAA in diatoms – first steps towards understanding

In **Paper III**, ten diatom strains - related in various degrees ranging from the same species to different classes - as well as three cyanobacteria species known to produce BMAA (Cox et al., 2005; Esterhuizen and Downing, 2008; Spacil et al., 2010; Berntzon et al., 2013; Jiang et al., 2013) were analyzed for free and protein-associated BMAA. Even though the in-house validated method was used, the BMAA concentration in the protein-associated fraction showed a broad range, from 0.0080 to 2.5797 $\mu g \cdot g^{-1}$DW (Table 6). This supports the hypothesis of variability in BMAA concentrations may be due to biological reasons such as the phytoplankton group (i.e. cyanobacteria, diatom or dinoflagellate), strain and growing conditions (Banack et al., 2010a; Banack et al., 2010b ; **Paper II**).

So far, the biosynthesis, metabolism and function of BMAA in diatoms are still unknown as well as the environmental factors that can induce or reduce its production. As mentioned above, studies in cyanobacteria have shown a negative correlation of BMAA production with nitrate and ammonium availability (Downing et al., 2011; Scott et al., 2014). Moreover, experiments with cyanobacteria cultures supplemented with BMAA had reveal signs of nitrogen starvation (Downing et al., 2012; Berntzon et al., 2013) and proposed a mechanism of BMAA metabolism via the GOGAT cycle (Downing and Downing, 2016). However, in the former study by Downing and Downing (2016) cyanobacteria were exposed to 100 µM BMAA and in the other two previous studies the BMAA concentrations added ranged from 0.05 till 42 µM (Downing et al., 2011) and from 0.01 till 50 µM (Berntzon et al., 2013). Since, the natural BMAA concentrations in cyanobacteria range between the low µg/g and ng/g (Table 1) some doubts may be inferred.

The variable natural production of BMAA by cyanobacteria and diatoms has also been verified in **Paper III** (Table 6). Therefore, in the study described in **Paper IV**, the naturally occurring concentrations of BMAA were taken into consideration when the diatom species *Phaeodactylum tricornutum* and *Thalassiosira weissflogii* were exposed to 0.005, 0.05 and 0.5 µM BMAA.

The exogenously applied BMAA was rapidly taken up in a concentration and time dependent manner in the free BMAA fraction of *P. tricornutum* and *T. weissflogii*, $F(12, 40) = 3.86$, $p<0.001$ and $F(12, 40) = 8.15$, $p<0.001$, respectively (see **Paper IV**). Moreover, most of the BMAA detected in the diatom cultures was in the free form**,** as previously observed in cyanobacteria (Downing et al., 2012; Berntzon et al., 2013). However, the concentrations of free BMAA in the *T. weissflogii* cultures exposed to 0.5 µM BMAA were significantly lower than in the *P. tricornutum* cultures ($F(1, 13)= 17.75$, $p<0.01$). After a 24 h hypothetical uptake, in *P. tricornutum* cultures, the free BMAA concentrations were reduced during the following days, suggesting that BMAA was metabolized (see **Paper IV**), as also previously observed in cyanobacteria (Downing et al., 2012). In *T. weissflogii*, BMAA was also

incorporated into proteins in a concentration dependent manner, F (3, 56) = 35.00, p<0.001. This could be due to a continuous uptake and storage of exogenous BMAA in the proteins and/or a stimulation of BMAA production by the presence of BMAA in the media.

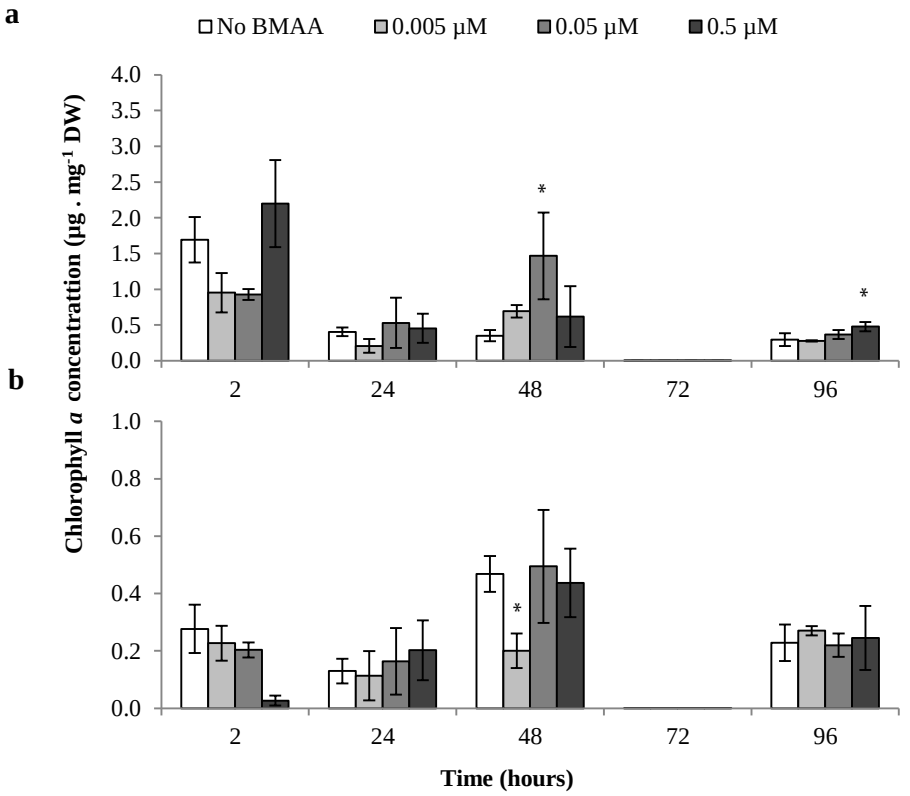

Figure 10. Chlorophyll *a* concentration (µg.mg^{-1} DW) in the cultures of (a) *Phaeodactylum tricornutum* and (b) *Thalassiosira weissflogii* exposed to three concentrations of exogenous BMAA (0.005; 0.05 and 0.5 µM), plus controls, over 96 h . Error bars represent the standard deviation of the mean, *n*=3. Significant differences in comparison to control cultures at each time point are indicated by an asterisk, * p<0.05. Note Y-axis scales of figure a and b are distinct; from **Paper IV**.

In **Paper III** the *P. tricornutum* cultures showed a response to added BMAA after 2 h of exposure. The levels of chlorophyll *a* in cultures exposed to 0.005 and 0.05 µM BMAA as well as protein content of all exposed cultures were reduced in comparison to the control cultures (Fig.10a and 11a), though not statistically significant. In the treated cultures of *T. weissflogii* a non-significant decrease in the protein content was only observed after 24 h (Fig. 11b). To some extent, these results mimic the *P. tricornutum* response to nitrogen deprivation i.e. progressively declining its chlorophyll *a* content becoming chlorotic (Alipanah et al., 2015; Levitan et al., 2015). During nitrogen deprivation a decrease in the abundance of proteins, especially the ones involved in nitrogen and protein metabolism, photosynthesis and biosynthesis

of chlorophyll has previously been seen (Hockin et al., 2012). However, the proposed nitrogen starvation signal of BMAA in diatoms was lower than previously observed in cyanobacteria (Downing et al., 2012; Berntzon et al., 2013). This could be because, in this study BMAA was supplemented to the diatoms in environmentally relevant concentrations, i.e. lower than in the cyanobacteria studies (Downing et al., 2012; Berntzon et al., 2013). Despite, or perhaps because of, diatoms were shown to contain higher concentrations of BMAA than cyanobacteria (Réveillon et al., 2015; Reveillon et al., 2016 ; **Paper III**), and might be better adapted to BMAA.

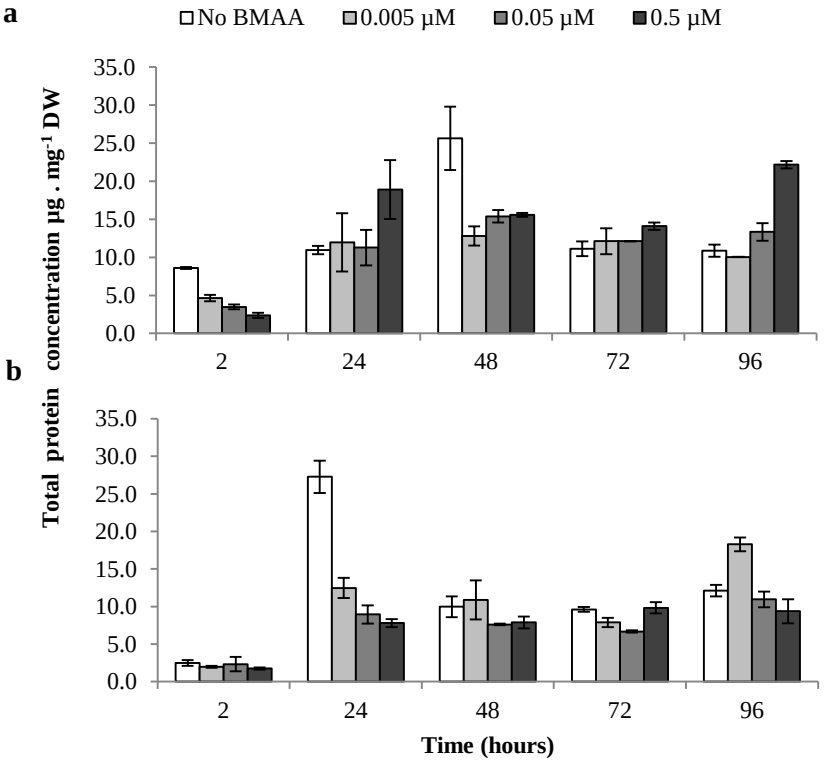

Figure 11. Total protein concentration (µg.mg^{-1} DW) in the cultures of (a) *Phaeodactylum tricornutum* and (b) *Thalassiosira weissflogii* exposed to three concentrations of exogenous BMAA (0.005; 0.05 and 0.5 µM), plus controls, over 96 h . Error bars represent the standard deviation of the mean, *n*=3; from **Paper IV**.

Interestingly and in accordance with the previously suggested hypothesis, in the *P. tricornutum* cultures exposed to 0.05 µM BMAA, extracellular ammonia was detected during the first 24 h. As well as in the *P. tricornutum* and *T. weissflogii* cultures exposed to 0.5 µM BMAA in all time points (see **Paper IV**). Diatoms normally do not excrete ammonia, but instead recycle it internally (Zehr and Falkowski, 1988; Allen et al., 2011), as it was observed in the control cultures, i.e. no ammonia detected. Hence, BMAA may interfere with the GS/GOGAT cycle in diatoms; as

previously been suggested for cyanobacteria (Downing and Downing, 2016). BMAA may inhibit the synthesis and/or stimulate the degradation of glutamine, subsequently induce glutamate release and thus signaling nitrogen depletion. As in cyanobacteria (Berntzon et al., 2013), an imbalance of glutamate/glutamine levels may also signal nitrogen starvation in diatoms. This since once entering the cell, nitrate might be reduced to nitrite and successively to ammonium (Allen et al., 2005; Bowler et al., 2010). Ammonium is assimilated to amino acids and other nitrogenous compounds by the joint action of GS/GOGAT (Zehr and Falkowski, 1988; Takabayashi et al., 2005). If GS/GOGAT is somehow disrupted, ammonium will start to intracellularly accumulate and possibly be excreted by the cell, as observed in the cultures of both diatom species exposed to 0.5 µM BMAA, and later re-assimilated. The re-assimilation is possible because diatoms, as cyanobacteria, possess a complete urea cycle (Armbrust, 2009). Accordingly, the concentrations of ammonia of both species cultures at the 96 h time point were at their minimal, in comparison with previous time points. The lack of glutamine could had led to the up-regulation of carbamoyl phosphate synthase, and then to the uptake of the available ammonia, which will enter the ornithine-urea cycle (Allen et al., 2011) and lead to the recovery of the diatom cells. Accordingly, the chlorophyll concentrations at the last experiment day were similar, though low, between treated and control cultures of both species (Fig.10). Moreover, the protein content of treated and control cultures was rather alike from 48 h to 96 h after BMAA exposure (Fig.11). Therefore, **Paper IV** suggests that the physiological role of BMAA might be, together with ammonia, to regulate/achieve a fast response of the diatoms to the rapid influx of nitrogen in an environment with constant nitrogen fluctuations.

Conclusions

This thesis contributed with knowledge in the following topics:

- **BMAA bioaccumulation in aquatic ecosystems**

Production and biotransfer of BMAA occurs not only in the brackish and marine ecosystems, but also in the limnic ecosystem, as shown here in Lake Finjasjön.

BMAA concentrations detected in fish from Finjasjön are equivalent to those previously reported in the Baltic Sea.

There was a positive correlation between concentrations of BMAA in fish brains and the factors species/feeding pattern and fish total weight.

Higher BMAA concentrations were detected in the brain of plankti-benthivorous fish with higher weight.

BMAA is heterogeneously distributed between fish tissues. It was found in 29% of the brain samples and in higher concentrations compared to in muscle tissue, where it was found in 16% of the samples and it was not at all detected in the liver or kidney.

The finding of both BMAA and mercury in muscle tissues of *Abramis brama* (bream) and *Sander lucioperca* (pike-perch) raises some concern in view of reported synergistic effects of these two toxic compounds (albeit at much higher concentrations than detected in this thesis).

- **The novel sources of BMAA**

The previous suggestion that benthic cyanobacteria might contain BMAA was not confirmed, but instead BMAA was for the first time detected in eukaryotic organisms, i.e. diatoms.

BMAA was detected both in field samples containing diatoms as well as in laboratory grown axenic diatom cultures.

Diatoms contain BMAA in higher concentrations than cyanobacteria and unlike in cyanobacteria, the toxin is found through consecutive diatom generations – which suggest that diatoms might be the main BMAA producers in aquatic ecosystems.

The BMAA concentrations were shown to be variable between diatom species and strains.

- **Evaluation of BMAA extraction methods**

The choice of extraction method was shown to be a cause of variation in measured concentrations of BMAA.

The method starting with extraction in 80% MeOH (used in **Paper I**) resulted in major losses at the SPE clean-up step, but without the SPE step, the linearity criterion was not fulfilled.

The method starting with extraction in 20% MeOH followed by acetone precipitation of proteins (used in **Paper II**) gave suitable linearity, accuracy and precision however, the recovery did not achieve validation criteria.

The first pellet of the "20% MeOH:acetone protein precipitation protocol" was found to contain high protein levels, and consequently protein-associated BMAA. Thus, if applying this method, this first pellet should not be discarded.

The method involving protein precipitation using TCA (later applied in **Paper III** and **IV**) was shown to meet in-house validation criteria.

Furthermore, the method evaluation showed that the TCA method does not require modified TCA concentrations or incubation time adjustments, to yield optimal protein precipitation.

The concentration of total protein, i.e. the ratio of protein-to-derivative (providing derivative in excess), was confirmed as essential to achieve a complete AQC derivatization of BMAA, and therefore accurate detection and quantification.

- **Physiological role of BMAA in diatoms**

The diatoms *Phaeodactylum tricornutum* and *Thalassiosira weissflogii* were found to react to exogenous BMAA added at the low concentrations of 0.005, 0.05 and 0.5 μM.

At 2 h, *P. tricornutum* had lower concentrations of chlorophyll *a* in the cultures where 0.005 and 0.05 μM BMAA was added and lower concentrations of protein in all treated cultures, in comparison to control.

All *T. weissflogii* cultures supplemented with BMAA had lower concentrations of protein at 24 h, in comparison to control.

Both diatom species excreted ammonium (measured as ammonia) in the culture media starting at 2 h after the addition of BMAA at 0.5 μM. In both species, the ammonia concentration was lower at the later sampling time point (96 h).

BMAA added to the cultures of *P. tricornutum* and *T. weissflogii* was taken up in a concentration and time dependent manner in the free BMAA fractions and the BMAA protein fraction of *T. weissflogii*.

The results suggest that BMAA may interfere with the diatoms GS/GOGAT cycle and that it may be closely associated with the cellular nitrogen balance in diatoms; thus, BMAA together with ammonium probably contributes to the fast response of diatoms to environmental nitrogen fluctuations.

Future research suggestions

Based on the results in this thesis, the following investigations are suggested:

- **BMAA bioaccumulation in aquatic ecosystems**

In order to find out which group or species of phytoplankton are the major producers of BMAA in freshwater, brackish and marine ecosystems, phytoplankton samples should be sampled over a year (covering all seasons) in these habitats. This sampling should be followed by species identification and quantification of species abundance and total BMAA. A statistical approach could then provide correlation indications between species and/or phytoplankton group and BMAA production.

A field bioaccumulation experiment comprising important commercial fish species and their preys and predators should be performed. So the percentage of BMAA retained in each trophic level could be quantified. A mesocosm study should also be carried out. Like this, both studies will complement each other and give reliable data that could later be used in modelling BMAA accumulation in aquatic ecosystems. Hence, the risk of consumption of BMAA-contaminated fish by human consumers could be predicted.

- **The novel sources of BMAA**

In view of the recent findings of BMAA producers among the diatom group, further species of this group should be tested for BMAA production. Therefore, investigate if as suggested for the cyanobacteria phylum, BMAA is also produced by almost all species of the diatoms phylum (i.e. Bacillariophyta).

- **BMAA extraction controversy**

An inter-laboratory validation of both extraction and analytical methods for BMAA should be promoted. Thus, if the scientific community working in BMAA research reached a consensus, all future BMAA studies could be directly compared.

- **Physiological role of BMAA in diatoms**

Monitor the effect of exogenous BMAA in diatoms at different molecular levels by a combined transcriptional and metabolite analysis, and thus come closer to an understanding of the physiological role of BMAA in diatoms.

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ba-gee-chi-ba-va…). To my stoical human, thank you for coping with me, especially in the final stages of this thesis, which made my quirkiness and grumpiness reach outrageous levels. I hope, besides many other things, to keep amusingly disagreeing with you far into the future! ☺ Lastly, I want to thank my parents for their unconditional support and love. Aos meus pais, obrigada por sempre acreditaram em mim, me acharem a melhor, mesmo não o sendo. Por isso, nunca deixei ou deixarei de tentar, não ser a melhor, mas fazer o melhor de mim. Obrigada!

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