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Neurotoxin BMAA And Its Isomeric Amino Acids In Cyanobacteria And *Spirulina* Food Supplements

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Cyanobacteria - Cyanotoxins

- Ancient group of photosynthetic microorganisms (3 billion years!!!)
- ✓ Significant contribution to the **creation of the oxygen** in Earth atmosphere!!!
- Also known as “blue-green algae”, generally distributed in the biosphere (lakes, ponds, rivers, brackish waters, sea)
- × Under favorable conditions they quickly multiply to form blooms and mats (Harmful Alga Blooms, HABs)
- × Several species and strains are toxigenic and produce toxic secondary metabolites (cyanotoxins)



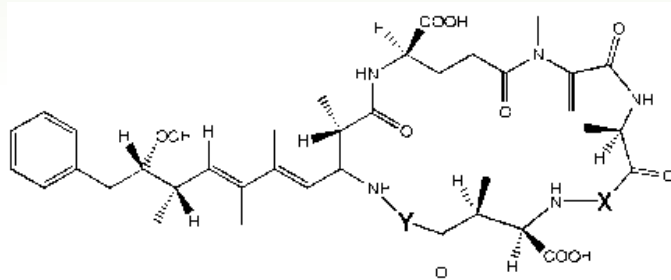
CYANOTOXINS

Cyclic Peptides	• Microcystins • Nodularins
Alkaloids	• Anatoxin • Cylindrospermopsin • Saxitoxins
Amino acids	• BMAA, DAB, AEG
Lipopolysaccharides (LPS)	

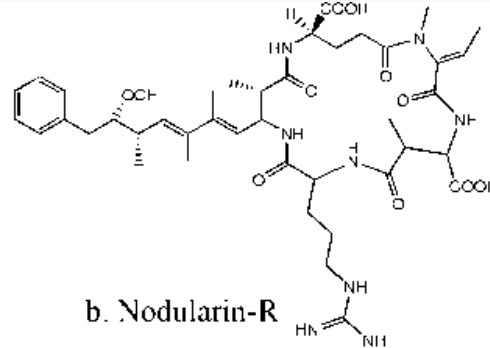


Cyanotoxins

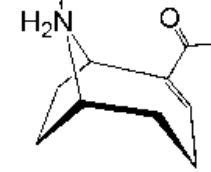
A large number of compounds with different chemical structures, physicochemical properties and toxic activities (**hepatotoxicity, neurotoxicity, or possibly carcinogenic**)



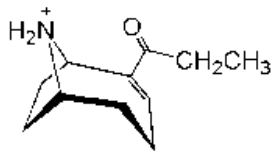
a. Microcystins



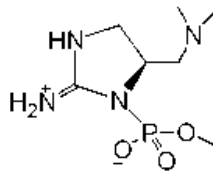
b. Nodularin-R



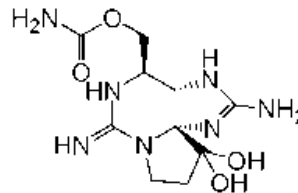
c. Anatoxin-a



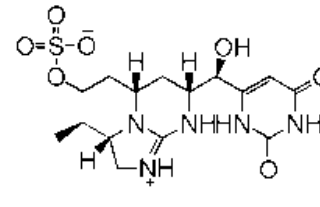
d. Homo-anatoxin-a



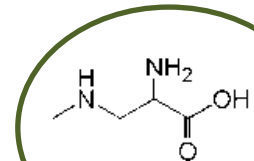
e. Anatoxin-a(S)



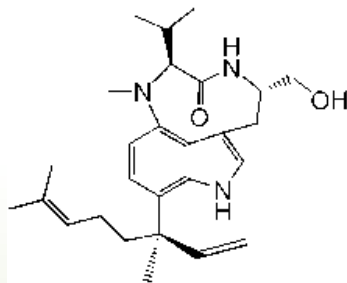
f. Saxitoxin



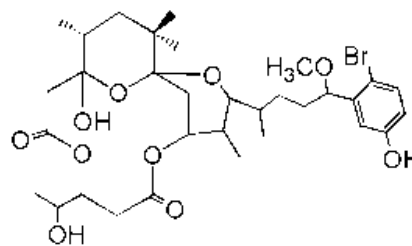
g. Cylindrospermospin



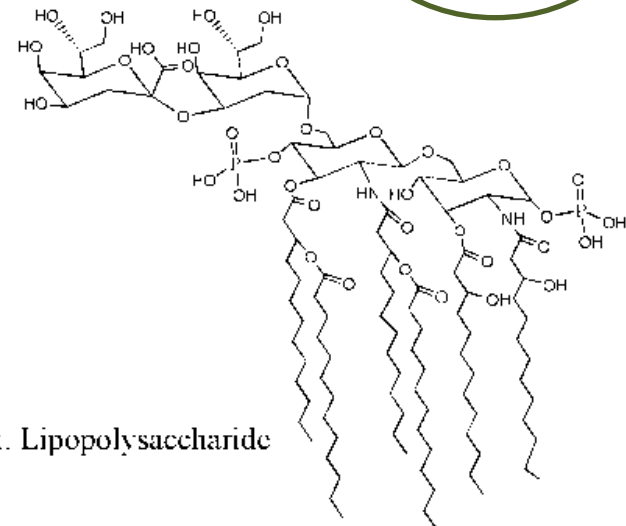
h. BMAA



i. Lyngbyatoxin-A



j. Aplysiatoxin

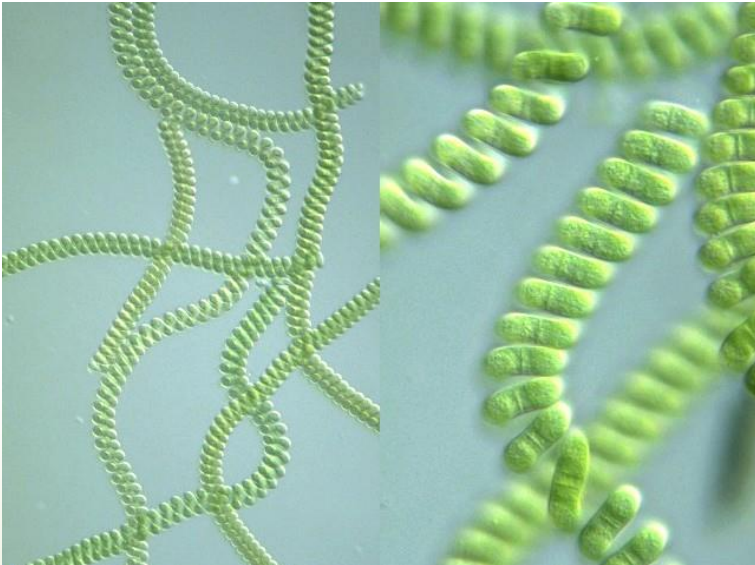
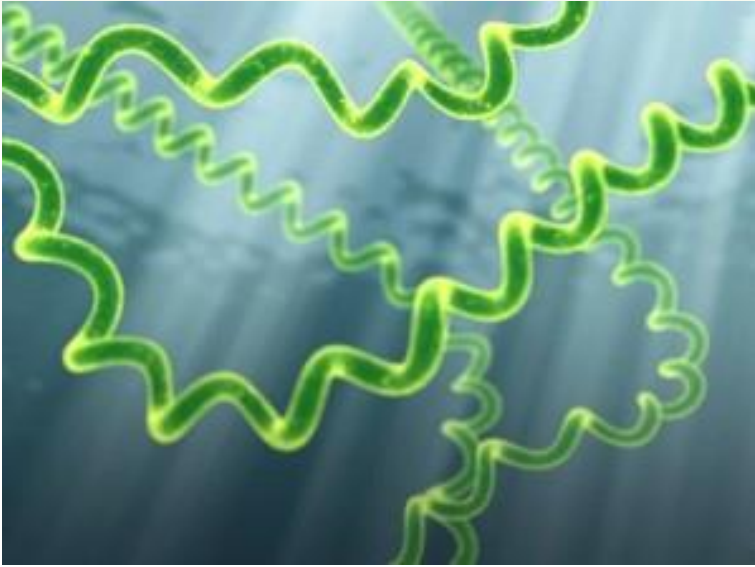


k. Lipopolysaccharide

Harmful Algal Blooms, HABs



SPIRULINA



Spirulina putative health benefits



- Reverse anemia (rich in iron)
- Anti-oxidant & Anti-inflammatory
- Lower LDL cholesterol and triglyceride levels
- Reduce blood pressure
- Help control blood sugar (diabetes)
- Relieve allergies



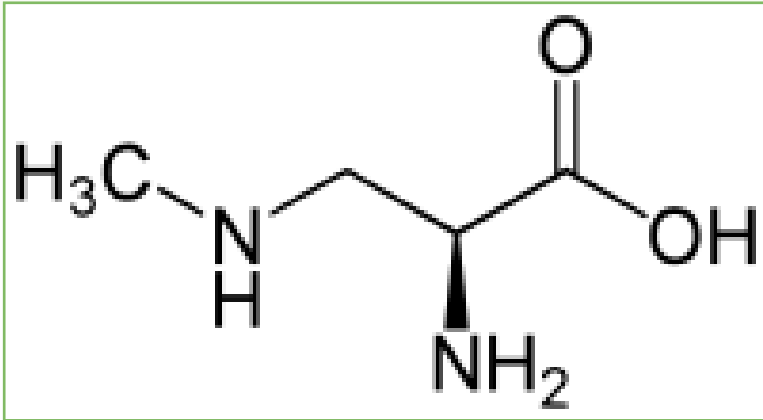
Caution for people with conditions such as

- Phenylketonuria (PKU) and
- Hemochromatosis (iron overload disease)



Is *Spirulina* only beneficial for the human health?

BMAA



β -N-methylamino-L-alanine

Properties

Non proteinogenic amino acid

M. Formula: **$C_4H_{10}N_2O_2$**

M. Weight: **118.1** g/mol

logP= **-3.77**

Relatively **polar** and **highly soluble** in water

Positive charged in acidic pH

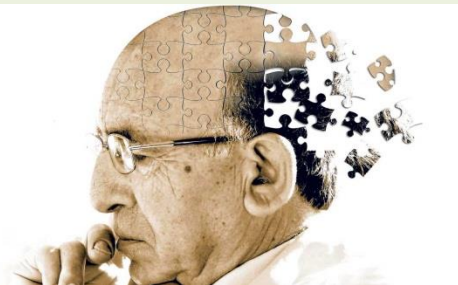
Stable (especially under acidic conditions)

Production

Free-living and **symbiotic cyanobacteria**

Axenic diatoms and **dinoflagellates** (eukaryotic organisms)

- BMAA can be present as **free** and **protein-bound**.



BMAA

Health effects

BMAA has been related to mechanisms causing:

oxidative stress, excitotoxicity and possible protein misfolding

These mechanisms are connected to Neurodegenerative Diseases

- **Amyotrophic Lateral Sclerosis (ALS)**

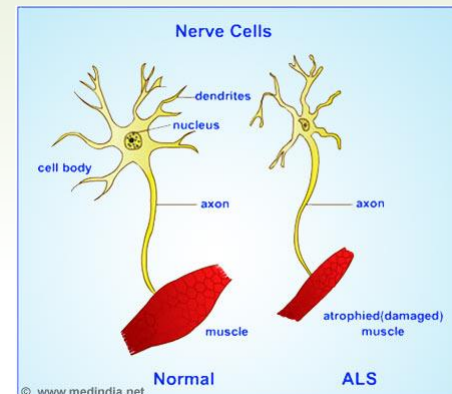
Progressive muscle weakness and atrophy

- **Alzheimer's dementia (AD)**

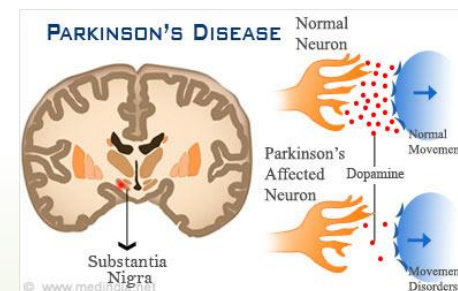
Loss of short-term memory, progresses to loss of language capabilities, disorientation, mood, ...

- **Parkinson's disease (PD)**

→ No available LD₅₀ or NOAEL,
no guideline has been proposed for BMAA

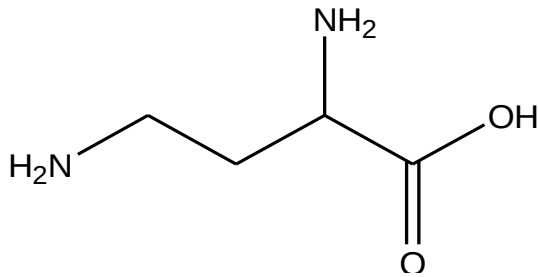


Healthy Brain Severe Alzheimer's



Isomers of BMAA

DAB



2,4-Diaminobutyric Acid

Properties

Non proteinogenic amino acids

M. Formula: **C₄H₁₀N₂O₂**

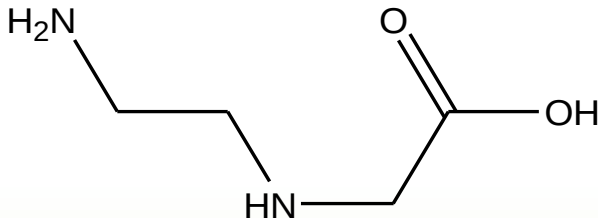
M. Weight: **118.1 g/mol**

logP= **-4.03 (DAB)** and **-3.97 (AEG)**

Relatively **polar** and **highly soluble**

Positive charged in acidic pH

AEG



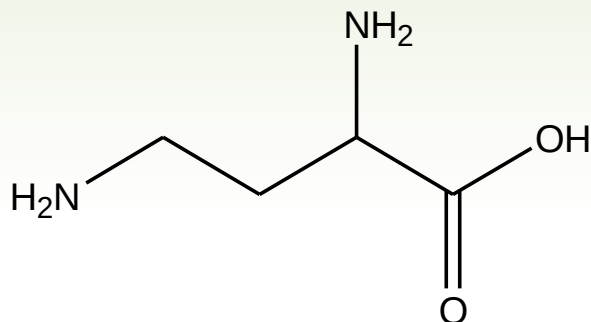
N-(2-Aminoethyl)Glycine

Toxicity and production

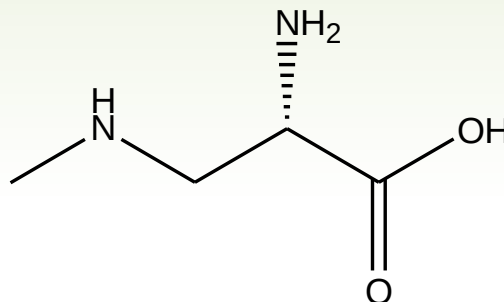
DAB has been indicated to be a **strong neurotoxic agent**, while it is believed to be a **hepatotoxic** as well. It is found in many **prokaryotic** and **eukaryotic** organisms.

AEG, has been claimed to be approximately **10,000** times less toxic than BMAA

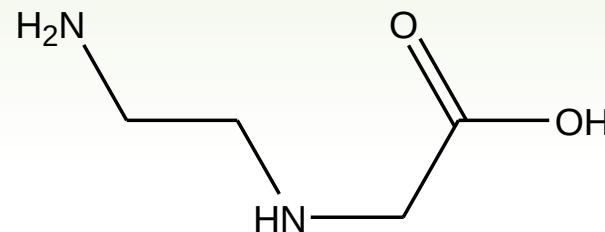
Challenges in BMAA analysis



DAB



BMAA



AEG

- **Low molecular weight** → There is a potential for significant background interferences in the lower range of the mass spectrum
- **Low concentrations** → Present in low concentrations in biological samples with complex matrices, which makes its identification and quantification a difficult task
- **Co - elution** → Structural isomers of BMAA (2,4-DAB, AEG, BAMA and others), might co-elute with BMAA during separation by HPLC and potentially cause a **false-positive result**

The BMAA debate

- Reported BMAA concentrations in cyanobacteria **vary widely between studies**.
- **Analytical methods** is a **major** source of differences between studies.
- **LC-FLD** and **LC-MS(/MS)** with or without derivatization (e.g. **AQC**) are commonly used.

Possible sources of error:

- Limited selectivity of LC-FLD
- Efficiency of derivatization
- Inadequate separation from DAB, AEG and other amino acids
- Loss of signal by ion suppression
- Method performance data (validation)

Inter-laboratory validation of a method proposed on a collaborative workshop (*Faassen et al. 2016*)

- ✓ Increased consensus on the most suitable analytical methods for BMAA analysis
- × All the possible sources of error → Challenges continue to exist
- Inter-laboratory validation of a recently evaluated protocol for BMAA analysis:

Faassen et. al, *“A Collaborative Evaluation of LC-MS/MS Based Methods for BMAA Analysis: Soluble Bound BMAA Found to Be an Important Fraction”*, **2016**, Marine drugs.



COST Action ES 1105 “CYANOCOST

- *Cyanobacterial blooms and toxins in water resources:*
 - *Occurrence, impacts and management”*

Analytical steps

✓ **Selection of Identification / Quantification criteria**

Tandem mass spectrometry (MS/MS)

Triple Quadrupole technology



✓ **Development of a method for Chromatographic separation**

Liquid Chromatography (LC)

(with the use of deuterated internal standards - d_3 -BMAA and d_3 -DAB)



✓ **Sample preparation**

Extraction, Hydrolysis



✓ **Sample analysis using LC-MS/MS (HILIC-ESI-MS/MS)**

Identification/Quantification of compounds using MS/MS

Analyte	t _R (min)	Precursor ion (m/z), [M+H] ⁺	Product ions (m/z)
BMAA	14.4	119	102 76* 88*
d3-BMAA		122	105 47* 76
DAB	17.2	119	101* 56* 74
d3-DAB		122	104* 105 59
AEG	20.5	119	102* 56 41

Identification points

Retention time

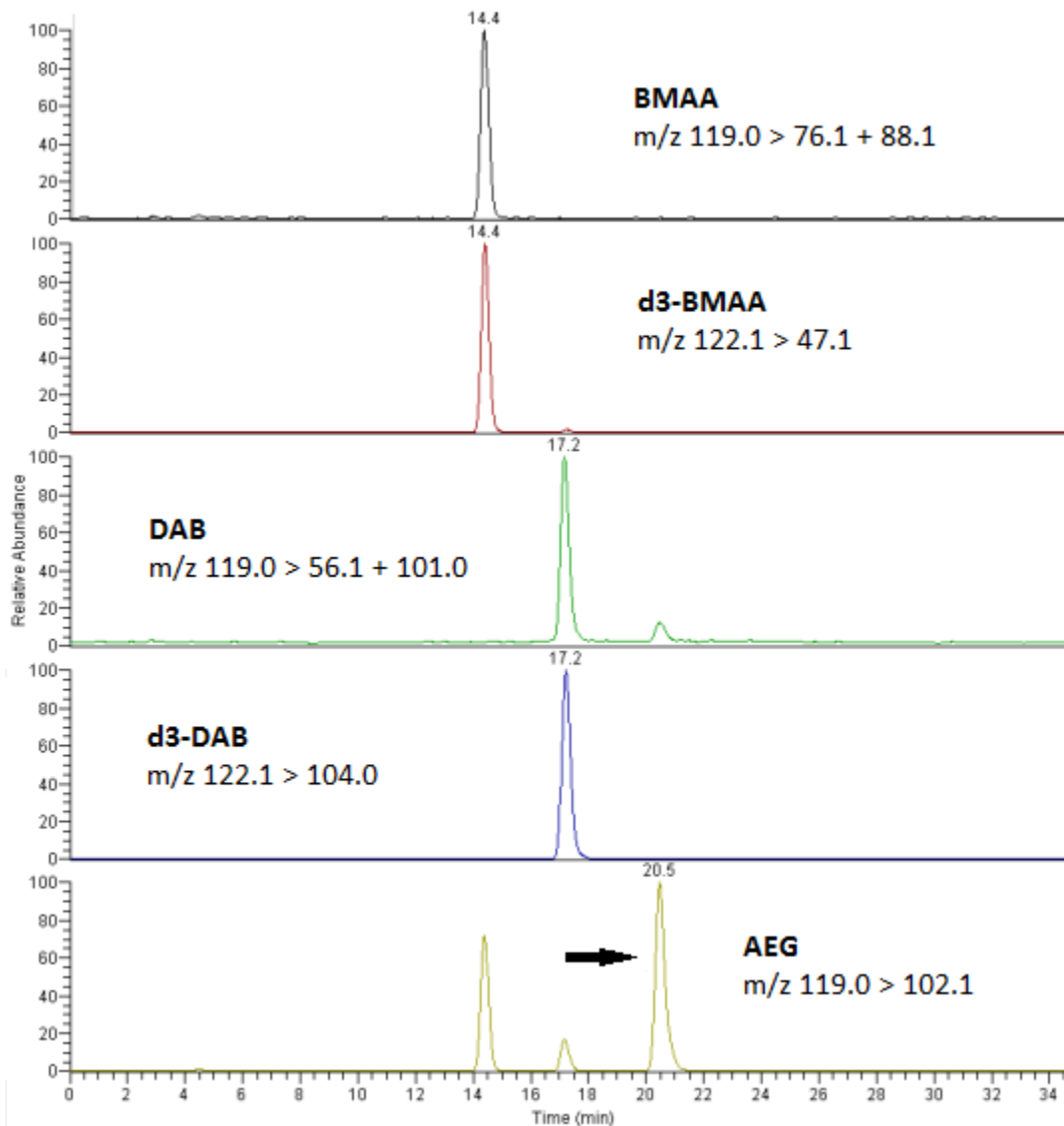
m/z precursor ion

m/z product ions

Ratio of m/z
qualification/quantification ions

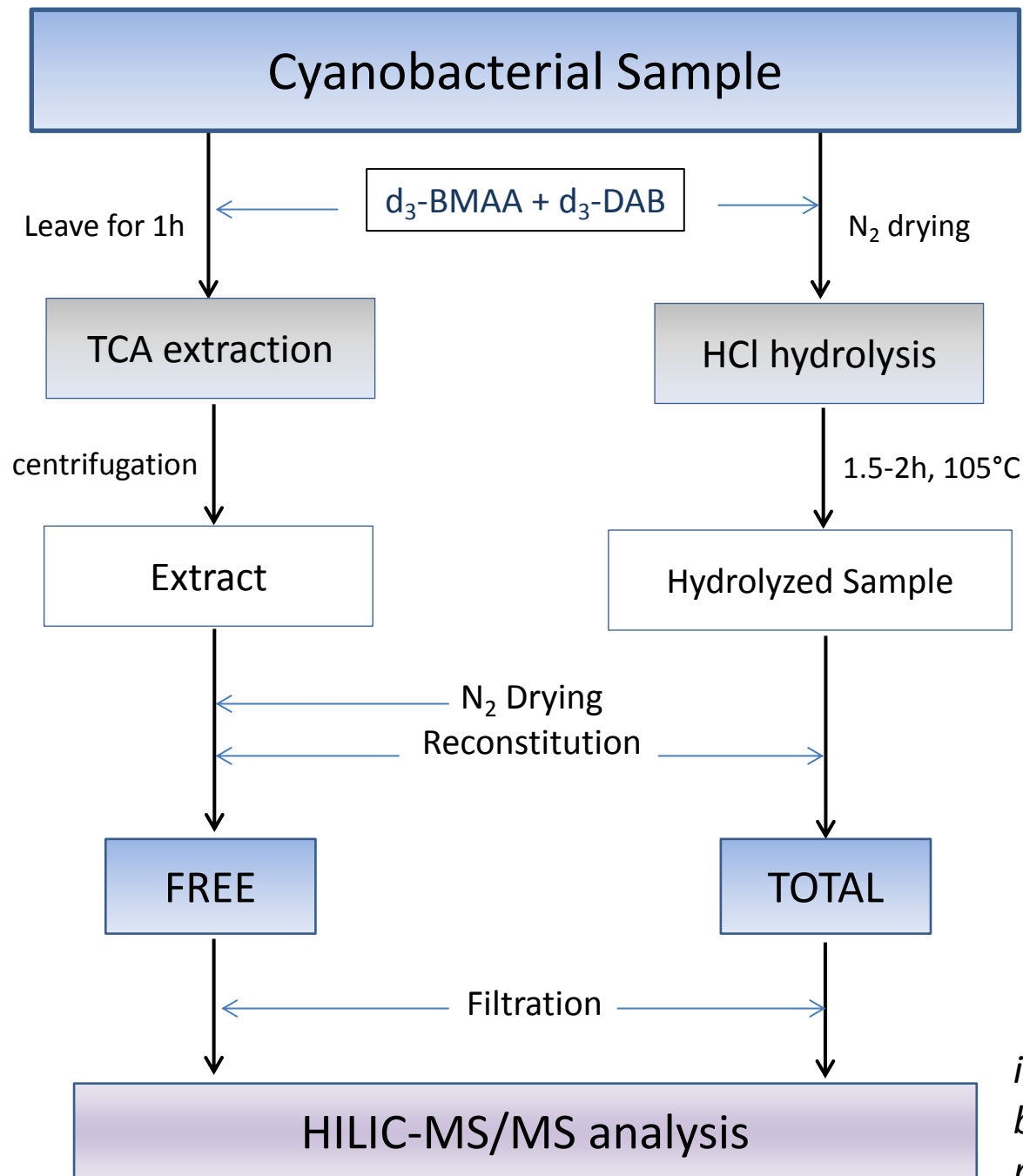
* Quantification ion(s)

Chromatographic separation of BMAA, DAB, AEG with HILIC-MS/MS

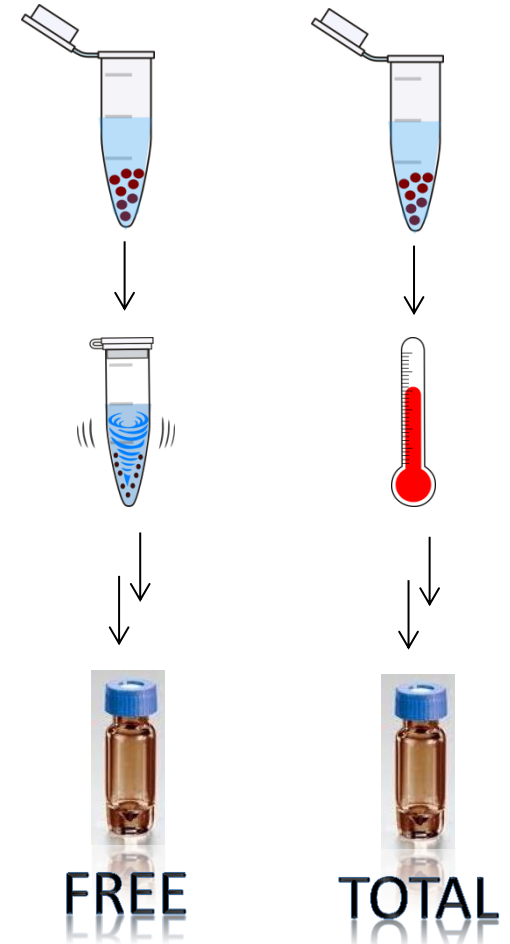


- Column: ZIC-HILIC, 2.1 x 100mm, 3,5 μ m, 100Å
- Gradient elution with (A) H₂O and (B) ACN both containing 0.1% HCOOH from 35% to 45% A
- Flow rate: 0.2ml/min
- Injection Volume: 5 μ l

MRM Chromatogram obtained from a standard solution of a mixture of BMAA, d3-BMAA, DAB, d3-DAB and AEG at the concentration of 100 μ g/L



Sample Preparation



in-house validation of a method based on a recently proposed protocol [Faassen et al., 2016].

Method Performance

Method Performance at Concentration level 10 µg/g			
Analyte →	BMAA	DAB	AEG
%Recovery¹	116.9	98.5	76.0
Repeatability (RSD%)²	6.0	2.6	5.2
Reproducibility (RSD%)³	6.8	5.9	7.3
Matrix Suppression (%)⁴	15.2	14.8	20.1
LOD (µg/g, d.w.)	0.4	0.5	0.5

¹ Mean % Recovery, N=12

² Relevant Standard Deviation under repeatability conditions, N=6

³ Relevant Standard Deviation under reproducibility conditions, N=12

⁴ Mean % Matrix suppression, N=12, spiking level 10µg/g

Application to real samples (1/2)

SPIRULINA FOOD SUPPLEMENTS

List of the commercially available “*Spirulina*” food supplements from the **Greek** market

Code	Type	Cyanobacteria listed on the product label	Place of Harvest	Origin of Manufacturing Company
SP1	tablet	“Spirulina”	Greece	Greece
SP2	powder	“Spirulina”	Greece	Greece
SP3	powder	Spirulina Platensis	China	N.A.
SP4	powder	“Spirulina”	China	Greece
SP5	tablet	“Spirulina”	Greece	Greece
SP6	tablet	“Spirulina”	N.A.	Greece
SP7	tablet	Spirulina Pacifica (Spirulina platensis/Arthrospira platensis)	Hawaii	The Netherlands
SP8	capsules	Spirulina (Arthrospira) Platensis	Greece	Greece
SP9	tablet	Spirulina Platensis	N.A.	U.K.
SP10	tablet	Arthrospira Platensis	Hawaii and N.A.	U.S.A.

Application to real samples (2/2)

CYANOBACTERIA

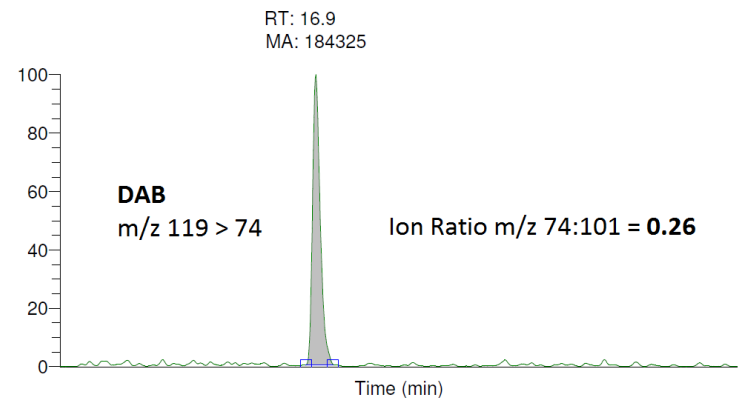
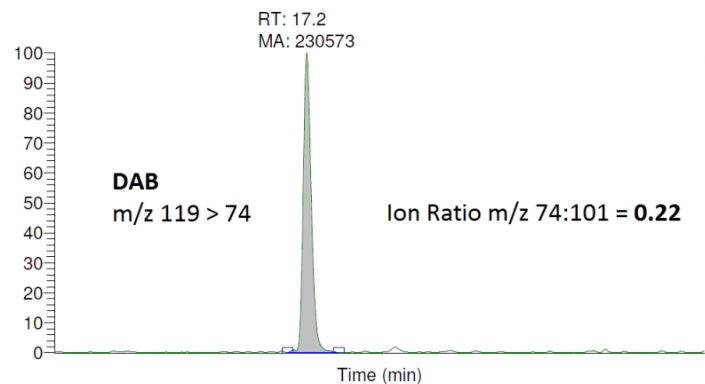
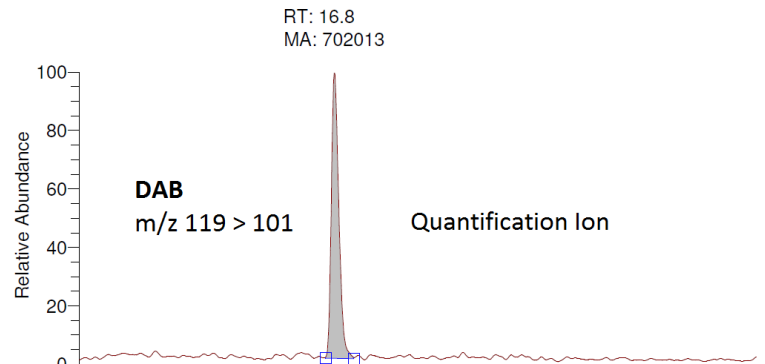
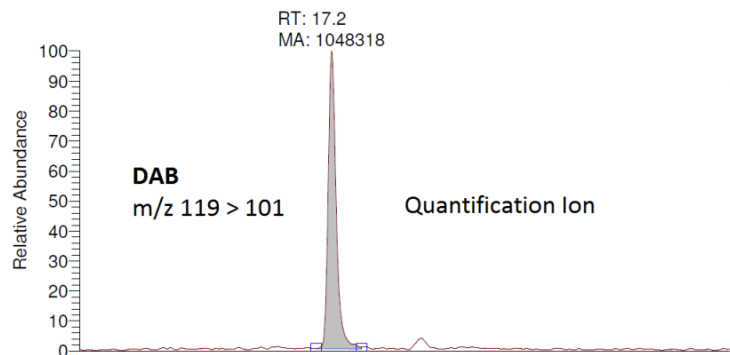
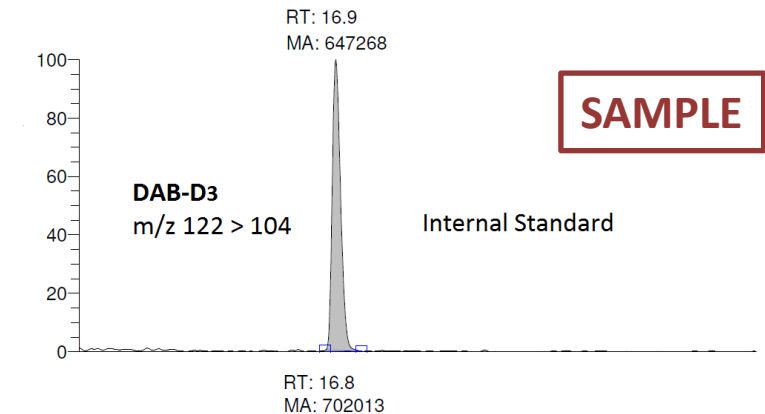
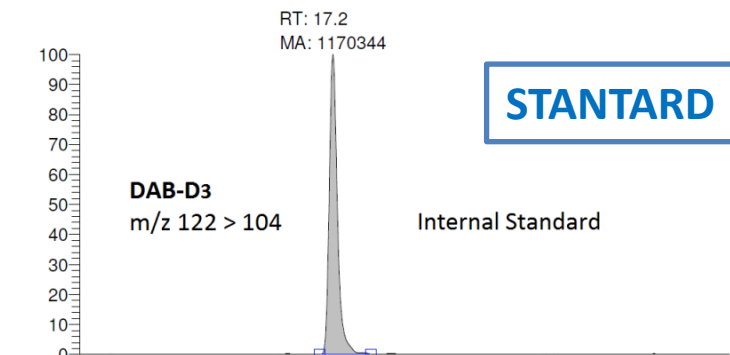
List of the lyophilized biomass obtained by **Greek** Lakes

Code	Lake	Location	Sampling date
L1	Kastoria	Macedonia, Northwestern Greece	2014
			2015
			2016
L2	Zazari	Florina, Northwestern Greece	2014
L3	Small Prespa (Mikri Prespa)	Florina, Northwestern Greece	2014
L4	Pamvotis	Ioannina, Northwestern Greece	2014
L5	Karla	Thessaly, Central Greece	2015

Unambiguous Identification of DAB

Based on Internal Standard and Ion Ratio Confirmation

Example: Commercially available Greek Biospirulina



Presence of FREE Neurotoxic Amino Acids in Cyanobacteria

Code	Identified cyanotoxin	Concentration (µg/g, d.w.)
Spirulina Food Supplements		
SP1	DAB	6.1
SP2	traces of DAB, AEG	< 0.5
SP3	DAB	0.5
SP4	DAB	0.8
SP5	DAB	0.6
SP6	Traces of DAB	< 0.5
SP7	DAB	0.6
SP8	DAB	0.7
SP9	DAB	0.6
SP10	DAB	2.2
LAKES		
L1	Traces of DAB	< 1.0
L2		
L3		
L4	Traces of AEG	
L5		

Conclusions

- ✓ Sensitive method with **low Limits of Detection** (LODs)
- ✓ Efficient chromatographic **separation** of the compounds
- ✓ High **recoveries** (%R)
- ✓ **Successful application to real samples**
 - Cyanobacteria from Greek lakes and
 - Spirulina Food Supplements obtained from the Greek market
- ✓ Identification of **DAB** in most of the *Spirulina* Food Supplements and confirmation with deuterated standard.
- ✓ Low concentrations, in accordance to the reported data in the literature



- *A work in progress*
- Further assessment of the occurrence of BMAA, DAB and AEG in cyanobacterial cultures and *Spirulina* dietary supplements

Acknowledgements

16th International Conference on Chemistry and the Environment (ICCE 2017)



COST Action ES 1105 “CYANOCOST

- *Cyanobacterial blooms and toxins in water resources:*
 - *Occurrence, impacts and management”*

Thank you for your attention!

