THE DEVELOPMENT OF AN ANALYTICAL SYSTEM FOR β-N-METHYLAMINO-L-ALANINE AND INVESTIGATION OF DISTRIBUTION OF PRODUCING ORGANISMS AND EXTENT OF FRESHWATER CONTAMINATION

Report to the
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by

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EXECUTIVE SUMMARY

Background and motivation for research

At the time of initiation of this project two publications had reported beta-N-methylamino-L-alanine (BMAA) production by free-living cyanobacteria (Cox et al., 2005; Banack et al., 2006) with one of these being marine cyanobacteria. Despite efforts by several laboratories internationally, these findings were not confirmed. However, BMAA had been identified as a potential risk to human health as it is implicated in Alzheimer's disease, Parkinsonism and Amyotrophic Lateral Sclerosis (ALS) (Murch et al., 2004a; 2003; Charleton et al., 1992; Banack et al., 2003). The possibility of sustained exposure to BMAA via drinking water supplies prompted the establishment of local analytical capacity for the neurotoxin, urgent verification of the production of BMAA by free-living cyanobacteria, the evaluation of the distribution of BMAA producing free-living cyanobacteria in South Africa and the extent of BMAA contamination of surface waters.

Beta-N-methylamino-L-alanine is intracellular in cyanobacteria, however with senescence it is likely that the BMAA is released into the surrounding environment. In the case of freshwater cyanobacteria BMAA would therefore be released into raw water which may be used as a potable water source. The efficacy of current treatment systems for BMAA removal is unknown. The potential risk to consumers was therefore also unknown but could be determined by addressing the extent of raw water contamination and treatment efficacy. Since biotoxins are often present in low concentrations, large volumes of water needed to be concentrated to be able to quantify BMAA. In order to assess the extent of free BMAA contamination of water, a concentration method needed to be developed.

Objectives

The stated aims of the proposal relating to BMAA research were:

- To establish and validate the method to test for free and bound Beta-N-methylamino-L-alanine (BMAA) in water and cyanobacterial bloom samples
- To establish a method to concentrate environmental BMAA from large volumes of water for the purpose of analysis
- To initiate the formation of an axenic culture collection of South African cyanobacterial isolates and establish the ubiquity of BMAA production by cyanobacteria
- To determine the prevalence of cyanobacterially-produced BMAA in South African freshwater impoundments

Summary of the major results and conclusions

A culture collection of South African cyanobacterial isolates was established, expanded and maintained as part of this project. Over 100 impoundments were sampled over a period of two years and all culturable cyanobacterial isolates were made unialgal and maintained as live cultures in the collection. This collection served as a resource for evaluation of BMAA production within and between taxonomic groups. The extent and diversity of the culture collection was ideally suited to serve this purpose and therefore achieved the stated objective.

Beta-N-methylamino-L-alanine analysis was optimized using a commercial chloroformate derivatization kit (EZ:faast) with gas chromatography – mass spectrometry (GC/MS) and high performance liquid chromatography – mass spectrometry (LC-MS) analysis of derivatized amino acids. An in-house BMAA analytical system (LC-MS) was installed and validated using a chloroformate pre-derivatized BMAA standard (Sigma). The resulting molecule was quantified against three internal standards: homoarginine, methionine-D3 and homophenylalanine. The BMAA standard was derivatized in varying concentrations on one day and injected in triplicate to assess machine reproducibility and on three consecutive days in order to analyse derivatization reproducibility. From the information obtained a calibration curve was constructed based on the representative molecular ion (*m/z* = 333) for quantification. Multiple user, delayed derivatization

and delayed analysis assessments were conducted to verify the system and determine how robust the developed methods were. Analytical stability was excellent with insignificant variation in individual quantification runs on a given sample. Derivatization reproducibility across the concentration range used for the calibration curve was acceptable (n = 5; r^2 = 0.985). The GC-MS BMAA detection developed for this project (Esterhuizen and Downing, 2008) was 15 times more sensitive than previously published fluorescent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate-derivatized BMAA detection methods and the LC-MS BMAA detection method is 50-fold more sensitive. The lower limit of sensitivity for detection was below 100 pg per injection, and quantification was possible at 148 pg.

Beta-N-methylamino-L-alanine was detected in 97% of all culture collection strains examined. All taxonomic divisions contained BMAA producing strains. Beta-N-methylamino-L-alanine was observed in both the unbound and protein associated forms but to different extents in different strains. The ratio of free to bound BMAA appeared to remain constant for any given strain. No taxonomic or geographic basis for BMAA production or the ratio of free to bound BMAA was observed.

A concentration protocol based on a strong cation exchanger with hydrophobic interaction was developed for extraction and concentration of BMAA from raw water samples. Recovery of BMAA from sterile distilled water samples of either one or 20 L, spiked with between 23.8 ng L^{-1} and 1.14 ng L^{-1}, ranged from 90 to 115% indicating complete recovery (SD at sample concentrations = 8%).

Dam water collected during an *Anabaena* bloom was pre-filtered and concentrated as previously described. The sample was evaluated for the presence of extracellular BMAA. From 20 L of water, no BMAA was detected (less than 500 pg L^{-1}). Filtrates were then hydrolysed in a non-quantitative manner and tested for BMAA using LC-MS with pre-derivatization. BMAA found in filtrate was in excess of 17 µg L^{-1}, and possibly as much as 40 µg L^{-1} intracellular BMAA. Twenty-five litres of water from a second dam were concentrated and tested for the presence of BMAA. Again no BMAA could be detected. A third dam with a *Microcystis* bloom also contained no extracellular BMAA in two separate samples collected from the dam on two separate occasions. Biomass collected from the dam was positive for BMAA on both occasions. These data suggest that BMAA is not released into the environment, or is rapidly degraded in, or removed from the environment.

The results described above constitute two peer reviewed publications, one of which appeared in 2008 and the other was submitted for publication.

The project yielded a reproducible and sensitive method for BMAA analysis from water or biomass and an efficient and simple method for concentration of BMAA from large water samples prior to analysis.

Capacity development

Three BSc Honours students completed their degrees in 2008. An MSc Student upgraded her degree to a PhD based on the results obtained as part of this project and completed her PhD in 2010. Two further MSc students have completed their work on this project and will graduate at the next available graduation ceremony. One PhD student has contributed to the project by molecular characterization of all isolates, and continues to work on these strains.

Recommendations

It is recommended that research on BMAA production, exposure, and health risks as well as possible remediation methods continues. It is further recommended that the analytical method developed here be adopted by all interested parties.

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2,4-DAB	2,4 diaminobutyric acid
ALS	amyotropic lateral sclerosis
BMAA	beta-N-methylamino-L-alanine
BSA	bovice serum albumin
ESI	electron spray ionization
GC-MS	gas chromatography – mass spectrometry
HARG	homoarginine
HPE	homophenylalanine
LC-MS	liquid chromatography – mass spectrometry
LC-MS-MS	liquid chromatography – tandem mass spectrometry
m/z	mass to charge ratio
PDC	Parkinsonism-dementia complex
SPE	solid phase extraction

INTRODUCTION

Beta-N-methylamino-L-alanine (BMAA) is a naturally occurring, non-proteinogenic amino acid that is an excitatory neurotoxin and results in neurodegeneration. It has been implicated in amyotrophic lateral sclerosis – Parkinsonism dementia complex (ALS/PDC). There has been considerable controversy over the analytical methods employed for BMAA detection and its role in ALS/PDC. Additionally, the controversy over analytical methods has resulted in some debate over the validity of the single report on cyanobacteria as the source of BMAA. This work therefore aimed at using a completely novel method for BMAA detection so as to evaluate the production of BMAA by cyanobacteria and determine the taxonomic extent of this production and the potential for human exposure to free BMAA in surface waters.

LITERATURE SURVEY

Naturally occurring BMAA is a neurotoxic non-proteinogenic amino acid reportedly produced by the majority of cyanobacterial isolates (Cox et al., 2005). Fatalities that occurred on Guam due to ALS/PDC were attributed to BMAA based on protein-associated BMAA found in the brain tissue of six ALS/PDC patients from Guam (Murch et al., 2004) and in one of two asymptomatic Chamorro individuals. In support of these data, no BMAA was found in the brain tissue of Chamorro fatalities from unrelated neurological diseases (Cox et al., 2003). The BMAA produced by symbiotic cyanobacteria (*Nostoc*) in *Cycas micronesica* coralloid roots (Cox et al., 2003) is consumed by Chamorro people in the form of flour products made from the cycad seeds, as well as by flying foxes (*Pteropus mariannus*). The flour was not considered to be an adequate source of BMAA as it was washed to remove free BMAA (Duncan et al., 1990). However, protein-associated BMAA was not taken into account (Murch et al., 2004). Flying foxes forage on the seeds of the cycads resulting in biomagnification of BMAA and exposure of consumers to elevated BMAA concentrations (Cox and Sacks, 2002; Banack et al., 2006). Montine et al. (2005), using an alternative analytical method, reported that no BMAA was detected in the brain tissue of Chamorro ALS-PDC patients. These non-replicated data from a single investigation cast doubt on the BMAA – ALS-PDC link. However, further evidence supporting the link between BMAA and ALS/PDC came in the form of the sudden decline in ALS/PDC occurrence on Guam with a decline in consumption of flying fox due to near extinction (Monson et al., 2003). Beta-N-methylamino-L-alanine was subsequently found in the brain tissue of Canadian Alzheimer's patients (Murch et al., 2004), suggesting alternate sources of BMAA in the food chain. Cox et al. (2005) found that culture collection strains of free-living freshwater and marine cyanobacteria from all five taxonomic sections contained BMAA and concluded that most, if not all, cyanobacteria produce BMAA. Cyanobacteria frequently found in drinking water sources have been found to produce BMAA therefore possibly increasing the risk of exposure to BMAA. However, the extent of exposure is currently unknown. Beta-N-methylamino-L-alanine has been shown to cause selective motor neuron demise at concentrations as low as 30 mM (Rao et al., 2006) and damage motor neurons at concentrations of 10 mM (Lobner et al., 2007).

Recent publications suggest that BMAA is not only present in cyanobacteria, but may also be released into water supplies and reservoirs used by humans (Metcalf et al., 2008; Banack et al., 2007). Metcalf et al. (2008) showed the co-existence of BMAA with other cyanobacterial toxins, which may result in synergistic effects. We therefore developed an alternative, robust, sensitive and highly discriminatory method for BMAA detection to investigate whether BMAA was produced by newly isolated cyanobacteria, and developed a solid phase extraction method for the concentration of BMAA from large volumes of water. The latter was deemed necessary because the safe exposure levels have yet to be determined and we sought sufficient sensitivity to ensure detection at any established level.

METHODS AND MATERIALS

Cultures representing taxonomic diversity and geographic distribution in Southern Africa were collected from various freshwater impoundments and made uni-algal by standard methods on BG-11 and BG-11_0 solid media (Rippka, 1988). Cultures were maintained in liquid media at a temperature of 23°C (±1°C) at a light intensity of 30-40 μmol.photon.m^{-2}.s^{-1} under constant illumination (Triton Dayglo ©). Culture purity was confirmed microscopically before BMAA analysis. Cyanobacterial cultures were centrifuged using a Beckmann Avanti J-20 centrifuge at 15 800 *g* at 4°C for 10 minutes to collect biomass which was snap-frozen in liquid nitrogen and lyophilized overnight in a VirTis bench top freeze dryer (condenser temperature of -51°C and a vacuum of 350 mtor). Beta-N-methylamino-L-alanine was extracted from fresh and lyophilized cultures (20-500 mg dry weight) by sonication (Bandelin sonoplus ultrasonic sonicator, 40% power at 1 × 30 second burst, and a 50% duty cycle pulse) with 0.1 M trichloroacetic acid. Free BMAA was obtained in the supernatant after centrifugation (Beckmann Avanti J-20 at 15,800 G for 3 minutes at 4°C) to precipitate proteins. Protein associated BMAA was released by liquid acid hydrolysis in 6 M HCl and 2% thioglycolic acid at 110°C for 24 hours in an inert atmosphere and filtered through a 0.22 μm filter (Lasec) before derivatization. Beta-N-methylamino-L-alanine was analysed using a Thermofinnigan Trace GC 2000 coupled to a Thermofinnigan Trace MS Plus with pre-derivatization of amino acids using Phenomonex EZ:faast™ or using a Shimadzu UFLC coupled to a Shimadzu MS (2010 EV) after derivatization using the LC form of the EZ:faast™ kit. For both versions the derivatization requires a concentration step on a proprietary sorbent medium, elution from, and removal of, the sorbent medium, and sample clean up as well as derivatization with a proprietary chloroformate derivative.

For GC-MS analysis a ZB-AAA 10 m × 0.25 mm Amino Acid Analysis GC Column was used to separate the compounds. Samples (2 μL) were injected using split injection at a ratio of 1:15 and an injection port temperature of 250°C. Helium was used as the carrier gas at a flow of 1.1 mL/min constant flow. Oven temperature was increased from 110°C to 320°C at 30° min^{-1}. The mass spectrometer ESI source (positive ion mode) and quadropole temperatures were set at 240°C and 180°C respectively with an auxiliary temperature of 310°C. Ion scan range was between 30 and 400 *m/z* with a sampling rate of 2^2 (3.5 scans s^{-1}). Data was analyzed using Excalibur software. The GC-MS system was validated using a range of dilutions of an authenticated BMAA standard (Sigma), negative controls as well as sample spiking with an authenticated standard. Bovine serum albumin (BSA) (Sigma) was used as a negative control for protein associated BMAA. BSA was hydrolyzed as described, pre-derivatized and analyzed. *Escherichia coli* was used as a negative control for non-protein associated cellular BMAA. Extraction, derivatization and analysis were as for cyanobacterial samples.

For LC-MS analysis samples were derivatized as described by Esterhuizen and Downing (2008) using the EZ:faast™ amino acid analysis kit for LC-MS (Phenomenex). Beta-N-methylamino-L-alanine was separated from other amino acids by liquid chromatography on a commercial column (Phenomenex AAA-MS 250 × 2.0 mm amino acid analysis column) using a LCMS 2010 EV (Shimadzu). A solvent gradient was used with A: 10 mM ammonium formate in water and B: 10 mM ammonium formate in methanol (0.0 min = 68% B, 13.00 = 83% B, 13.01 = 68% B, 17.00 68% B) at a flow rate of 0.25 min/min and 1 μL sample injection volume. Column temperature was kept constant at 35°C. The mass spectrometer ESI source (positive ion mode) temperature was set at 250°C. The ion scan range was between 20 and 600*m/z*. The detector voltage was set at 1.5 kV. The interface voltage was set at 4.5 kV and the CDL voltage at -20 V with the heating block at 200°V. Data was analyzed using LCMS solutions Ver. 3 software. The liquid chromatography-ass spectrometry (LC-MS) system was validated using a range of dilutions of an authenticated BMAA

standard (Sigma), negative controls as well as spiking 20 standard amino acids with the BMAA authenticated standard**.**

For recovery and concentration from large volumes of water Strata-X-C 33 µm polymeric strong cation sorbent gigatubes (Phenomenex) were conditioned with 20 mL of pure methanol (LC grade, Merck) at a flow rate of 20 mL/min. This was followed by an equilibration step using 20mL of PBS buffer (pH 4) at a flow rate of 20 mL/min.

The BMAA (23.82 ng) was added to 1 L of sterile reverse osmosis (RO) purified water and concentrated using the solid phase extraction described at a flow rate of 30 mL per minute where after it was washed with 20 mL each of 2% formic acid and 100% methanol (10 mL/min) before elution in 5% ammonium hydroxide in a 1:1 ratio of methanol/acetonitrile (3 mL/min). The sample was then acidified and dried under a low nitrogen gas stream to remove hydroxide ions that may interfere with the derivatization. The sample was resuspended in 1 mL of sterile RO water. One twentieth of the eluant was derivatized and analysed on the LC-MS with pre-derivatization. A tenth of the previous BMAA sample (2.382 ng) was then similarly added to RO water and concentrated and analysed.

Testing was then scaled-up to investigate recovery in a larger volume. Beta-N-methylamino-L-alanine (23.8 ng) was added to 20 L of RO water and concentrated as described. Eluent collected was dried under a stream of nitrogen gas and resuspended in 1 mL of RO water and derivatized.

Samples (20 L) from three potable water supplies were pre-filtered with glass microfibre filters (GFA, Whatman) and concentrated as previously discussed. Total BMAA in collected bloom material was determined by hydrolysing the filtrate on the glass fibre filters in 6 N HCl with 2% thioglycolic acid at 110 °C for 24h in an inert atmosphere. Hydrolysed extracts were filtered through a 0.22 µm filter (Lasec) and the pH was corrected to between 1 and 2 with NaOH before derivatization.

RESULTS

Method Development and Evaluation

The initial phase of method development resulted in a GC-MS based BMAA quantification method (Esterhuizen and Downing, 2008). The lower limits of sensitivity of detection and quantification of

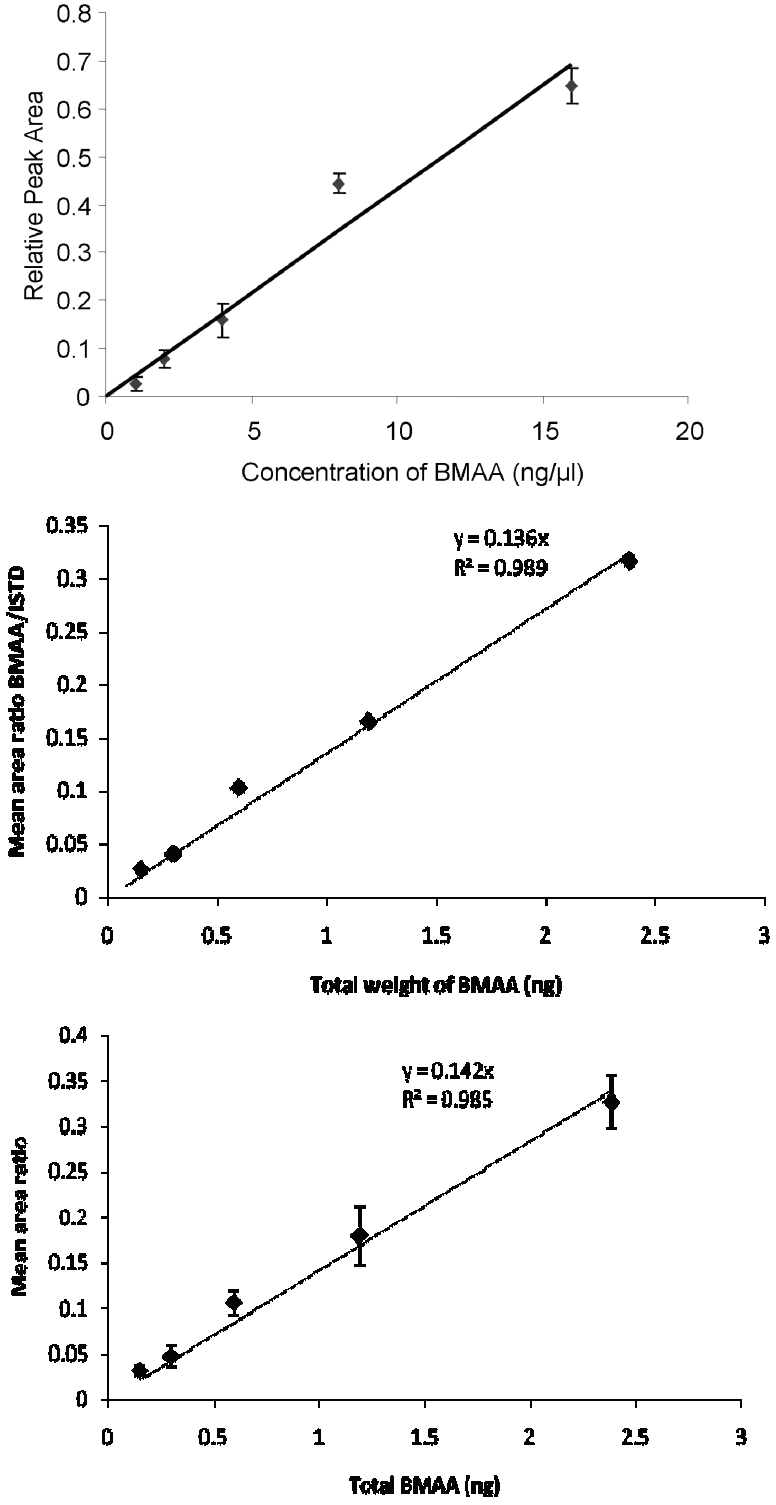

Figure 1: Calibration curve of BMAA standard against the internal standard methionine-d3. (A) is the standard curve obtained off the GC-MS, (B) shows the standard curve evaluating the Shimadzu LC-MS machine reproducibility (*n*=3, r^2=0.989) and (C) shows user validation for derivatization and analysis (*n* =3, r^2=0.985).

an authenticated standard were 0.0042 nmol per injection and 0.084 nmol per injection respectively. The lower limit of detection in a matrix of 20 µM each of the 20 standard amino acids was 0.0252 nmol per injection. The LC-MS method improved the sensitivity 10 fold.

Figure 1 shows the standard curves obtained using commercial BMAA standard on both mass spectrometers. Figure 2 shows typical chromatograms obtained for each analytical method. With analysis by GC-MS BMAA had a retention time of 3.95 minutes and a mass/charge ratio (*m/z*) of 130.2. LC-MS analysis yielded a peak at retention time of 9.4 minutes with a molecular ion *m/z* of 333. Some variation in retention time within complex matrices was observed.

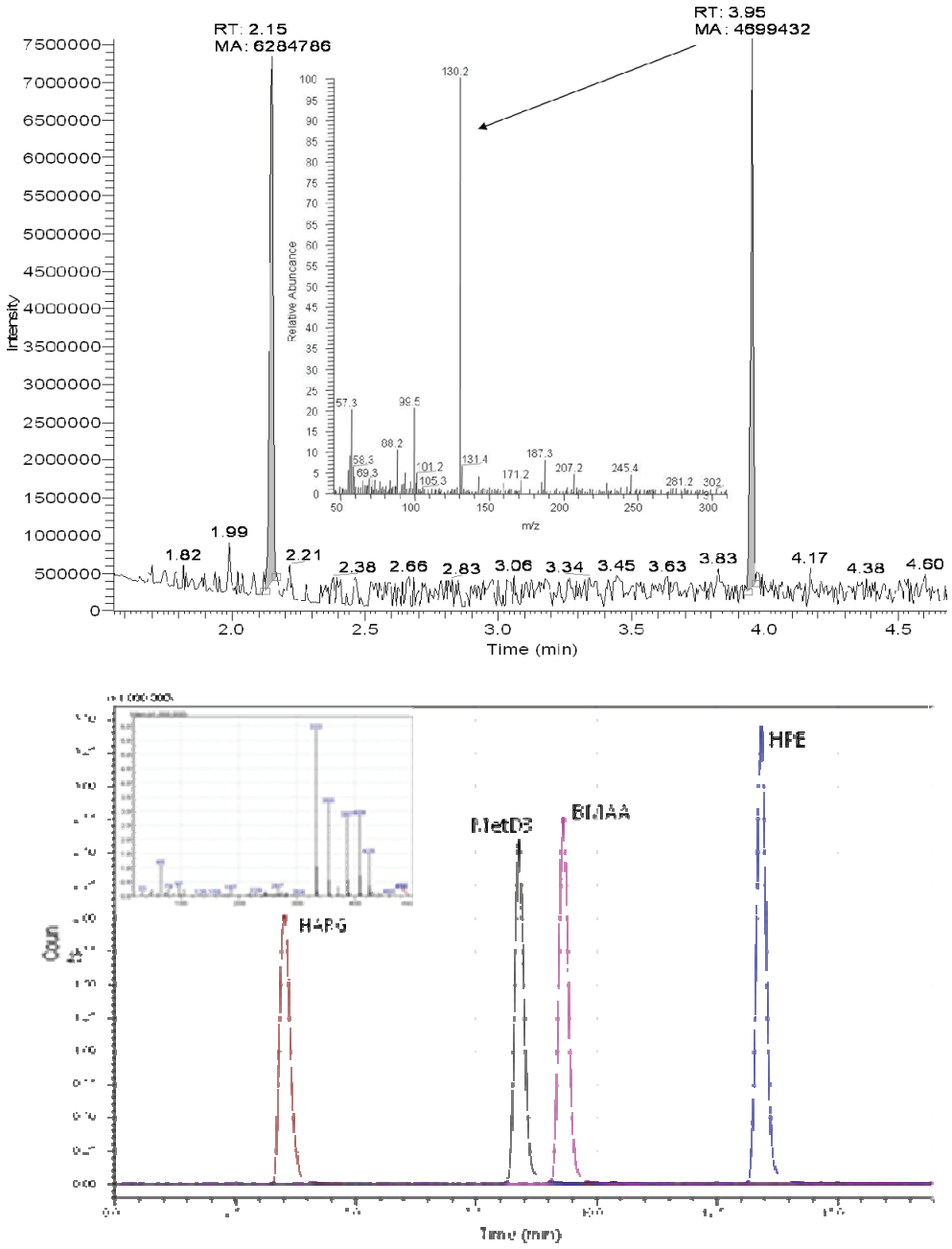

Figure 2: Typical chromatograms of isolates. (A) GC-MA chromatogram (insert is mass spectrum at the retention time of the BMAA standard (Esterhuizen and Downing, 2008). Note the 130 *m/z* molecular ion. (B) LC-MS of the entire run showing only the selected *m/z* chromatograms of the internal standards and BMAA (insert shows mass spectrum at BMAA elution time). Internal standards are HARG homo-arginine (*m/z* 317), MetD3 methionine D3 (*m/z* 281) and HPE homo-phenylalanine (*m/z* 308)

Despite controversy regarding the validity of BMAA detection methods and possible misidentification of isomers of BMAA, particularly 2,4-diamino butyric acid (2,4-DAB) (Krüger *et al.,* 2010) (Figure 3), the method presented here clearly distinguished between isomers (Figure 4).

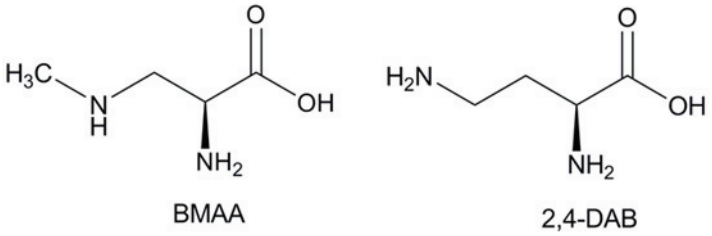

Figure 3: BMAA and 2,4-diamino butyric acid (2,4-DAB).

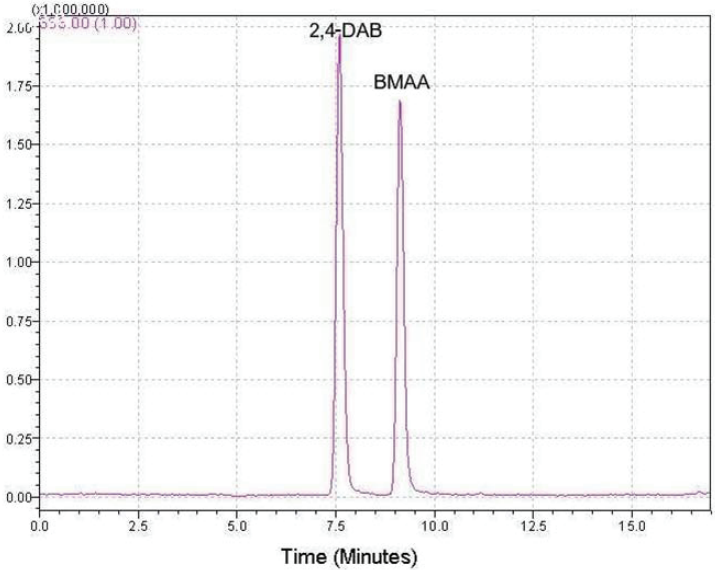

Figure 4: Shimadzu 2010EV LC-MS single quadrupole detection of separated propylchloroformate derivatized BMAA and 2,4-DAB using a 250 × 2.0 mm EZ:faastTM AAA LC column (Phenomenex).

Krüger *et al.,* 2010 also suggest that tryptophan, the propyl chloroformate derivative of which shares the same *m/z* with BMAA, could lead to false positive BMAA results. Figure 5 clearly shows the chromatographic separation of tryptophan from BMAA.

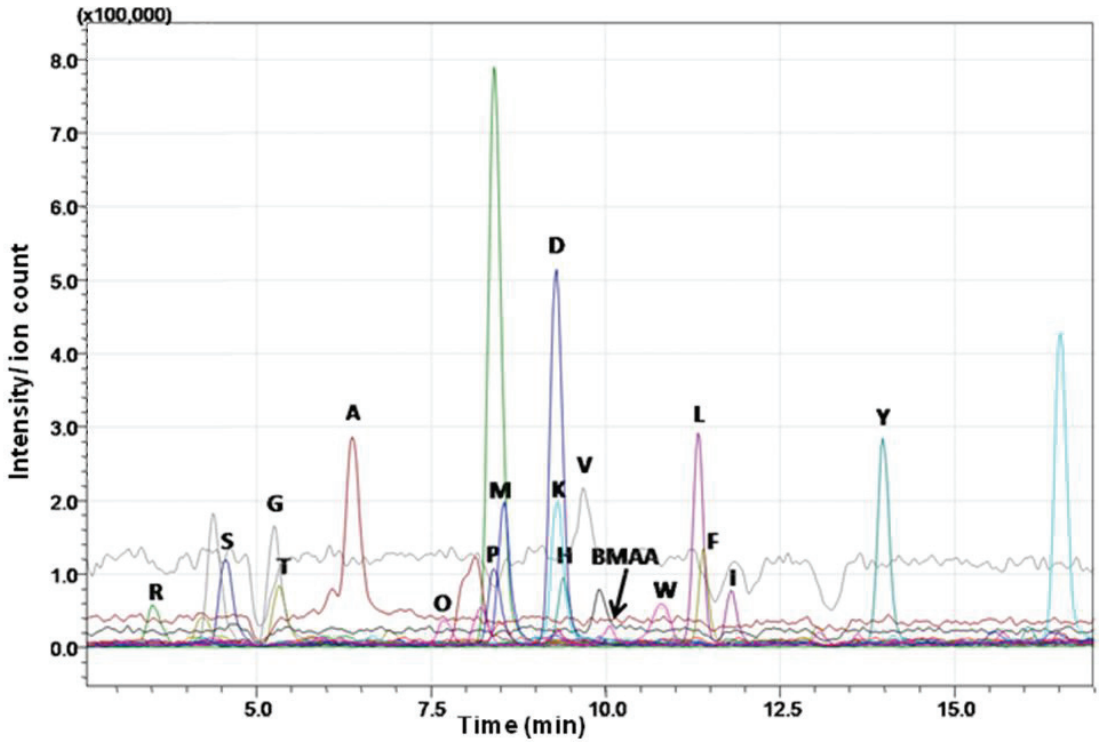

Figure 5: SPE recovery of BMAA from an amino acid matrix, detected using a Shimadzu 2010EV LC-MS single quadrupole for propylchloroformate derivatized amino acids. Samples were derivatized as described by Esterhuizen and Downing (2008) using the EZ:faastTM amino acid analysis kit for LC/MS (Phenomenex). Beta-N-methylamino-L-alanine was separated from other amino acids by liquid chromatography on a commercial column (Phenomenex AAA-MS 250 × 2.0 mm amino acid analysis column) using a LC/MS 2010 EV (Shimadzu). A solvent gradient was used with A: 10 mM ammonium formate in water and B: 10 mM ammonium formate in methanol (0.0 min = 68% B, 13.00 min = 83% B, 13.01 min = 68% B, 17.00 min = 68% B) at a flow rate of 0.25 min/min and 1 µL sample injection volume. Column temperature was kept constant at 35ºC.The mass spectrometer ESI source (positive ion mode) temperature was set at 250ºC. The ion scan range was between 20 and 600 *m/z*. The detector voltage was set at 1.5 kV. The interface voltage was set at 4.5 kV and the CDL voltage at -20 V with the heating block at 200ºC. Data was analyzed using LC/MS solutions Ver. 3 software. Detection by MS was as follows: Arginine (R) 303 *m/z*, Serine (S) 234 *m/z*, Glycine (G) 204 *m/z*, Threonine (T) 248 *m/z*, Alanine (A) 218 *m/z*, Ornithine (O) 347 *m/z*, Proline (P) 244 *m/z*, Methionine (M) 278 *m/z*, Lysine (K) 361 *m/z*, Aspartate (D) 304 *m/z*, Histidine (H) 370*m/z*, Valine (V) 246*m/z*..

Testing of South African Cultures

Nearly all tested cultures (97%) were reproducibly positive for BMAA although several were below the limit for quantification (Table 1). Only one of the tested strains showed an absence of BMAA in both the free and bound fractions. No relationship existed between the amount of free and bound BMAA. Beta-N-methylamino-L-alanine concentrations for these novel isolates from South African freshwater impoundments are lower than previously reported values (Cox et al., 2005) for culture collection strains. It should also be noted that BMAA concentrations varied considerably depending on whether extracted from fresh or lyophilized cultures. Five cultures where used for this comparison, all of which showed reproducibly higher BMAA concentrations when extracted from fresh culture.

Table 1 shows the BMAA content for a range of South African isolates from the cyanobacterial culture collection, representing both taxonomic and geographical diversity.

Table 1: The content of free and protein associated BMAA in cultures of free-living freshwater South African cyanobacterial isolates.

Cyanobacterial Strain/ Species	**Section** (Rippka 1988)	**Origin/ Catchment**	**Free BMAA (µg/g)**	**Bound BMAA (µg/g)**
Microcystis	I	PCC 7806	0.00	188.34
Microcystis	I	C811	0.135	NQ
Microcystis	I	D351	NQ	1.10
Synechocystis	I	PCC6803	0	NQ
Synechocystis	I	C522	0.126	0
Synechocystis	I	Merthley	0.391	1.290
Synechocystis	I	J341	0.23	5.17
Synechococcus	I	Q131	0.00	3.85
Cyanodiction	I	R202	22.75	ND
Leptolyngbya	III	B121	NQ	1.03
Leptolyngbya	III	D351	1.45	1.79
Leptolyngbya	III	J341	65.42	9.69
Leptolyngbya	III	Q411	0.05	2.91
Leptolyngbya	III	R203	NQ	0.10
Limnothrix	III	K901	NQ	2.05
Oscillatoria	III	C511	0.31	ND
Oscillatoria	III	D241	NQ	48.68
Oscillatoria	III	D351	57.49	ND
Oscillatoria	III	G401	146.88	ND
Oscillatoria	III	J121	0.00	21.77
Oscillatoria	III	V203	1.19	2755.61
Oscillatoria	III	C421	NQ	1.227
Oscillatoria	III	C521	0.976	0.936
Oscillatoria	III	C523	0	1.48
Oscillatoria	III	D231	NQ	0.0518
Oscillatoria	III	D237	0.058	0.179
Oscillatoria	III	D241	0.116	10.616
Oscillatoria	III	G405	0	0.751
Oscillatoria	III	J121	0.201	0
Planktothrix	III	D311	2.04	0.00
Planktothrix	III	H602	0.00	7.14
Planktothrix	III	S701	0.34	0.00
Phormidium	III	H602	NQ	14.91
Cyanobacterial Strain/ Species	**Section** (Rippka 1988)	**Origin/ Region**	**Free BMAA (µg/g)**	**Bound BMAA (µg/g)**
Pseudoanabaena	III	G227	0.00	3.19
Pseudoanabaena	III	K401	5.07	2.35
Pseudoanabaena	III	R203	0.00	8.99
Pseudoanabaena	III	A213	NQ	0
Pseudoanabaena	III	C321	0.876	NQ
Calothrix	IV	C511	1.00	0.00
Calothrix	IV	D242	0.050	0
Calothrix	IV	C522	0.060	NQ
Anabaena	IV	J241	0.00	0.56
Anabaena	IV	J331	0.00	0.00
Anabaena	IV	A224	0.156	0
Anabaena	IV	A311	NQ	0
Anabaena	IV	V202	0	0
Anabaena	IV	V203	0.577	1.393

*NQ Detected but not quantifiable

*ND Not determined

Development of a SPE method for quantification of BMAA in raw water samples

Results of BMAA recovery from spiked water are tabulated in Table 2 below. High BMAA recovery rates were achieved for BMAA in deionized water. However BMAA recovery decreased in the presence of other compounds competing for cation column binding on the recovery column or for binding during the solid phase derivatization procedure.

Table 2: BMAA recovery tests on Phenomenex Strat-X® SPE columns.

Sample Name/ Description	BMAA added (µg)	% Recovery
1 L	2.382	96
1 L	0.238	103
20 L	2.382	78
1 L amino acid matrix	2.382	46
1 L Salt matrix	2.382	63
1 L pond water	2.382	43

For deionized water the recovery rates were as expected from 1 L. The reduced recovery from 20 L was attributed to losses due to degradation or binding as previously observed (typically around 5% every 24 hours) and the long filtering time required for SPE of 20 L. Beta-N-methylamino-L-alanine recovery in an amino acid matrix resulted in a very low recovery rate either as a function of overloading of the SPE column or due to competition for derivatization. The specified column capacity was sufficient however, suggesting competition during derivatization as cause of the reduced yield. Similarly, the salt matrix resulted in reduced recovery presumably also due to competition for solid phase during derivatization. Given the sensitivity of the detection method, this is relatively simple to overcome for quantitative analysis from both complex organic and inorganic matrices by reducing the volume of sample. Losses from spiked pond water were also substantial despite a relatively low amino acid or salt load suggesting removal of BMAA by filtration of the pond water prior to SPE and derivatization.

The biomass from the filtered pond water was also tested and found to contain 159% of the spiked amount, yielding 199% of the total suggesting a biomass contribution of approximately 2.3 µg despite the lack of a bloom or scum. This suggests a high level of BMAA in surface waters generally and prompted evaluation of three surface potable water sources.

Evaluation of water bodies

No extracellular BMAA was detected in any of the surface waters tested. Water source 1 had no visible cyanobacteria bloom and the filtrate was largely inorganic. No BMAA was detected in the hydrolyzed filtrate sample. Water source 2 had an *Anabaena* bloom. Hydrolysed filtrate from this sample yielded between 17 and 40 µg BMAA per litre of sample (Figure 6.). Similarly, a *Microcystis* bloom on water source 3 contained 25 µg BMAA in the filtrate from one litre of sample.

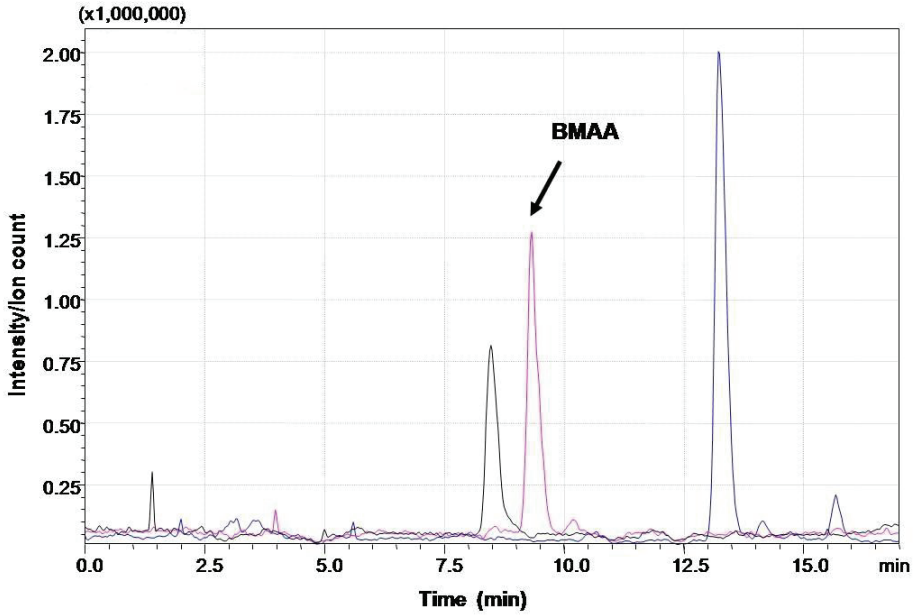

Figure 6: Chromatogram showing 333 *m/z* of BMAA extracted from biomass filtered out of an Anabaena bloom and two internal standards.

The presence of BMAA in the biomass from these samples, and the absence of free BMAA in the water, suggests absence of BMAA transport out of the cells. However, cell lysis is a common event in blooms and the complete absence of BMAA at the sensitivities obtained with our LC-MS method, suggests rapid uptake of BMAA in the environment.

DISCUSSION

The derivatization and detection method described here is available as a standardized kit that is both simple and rapid as well as being extremely sensitive. Final sensitivity is equipment dependant but detection of BMAA at concentrations below 100 ng/L should be achievable based on an on-column sensitivity of 4.2 pmol per injection and the solid phase derivatization, elution, resuspension system which can concentrate up to 150 times. With a 2 µL injection this represents detection limits below 13 nM (or roughly 1.5 ug/L) in sample water. With the strong cation exchange SPE method developed here, sensitivity can be increased substantially but stability factors during concentration must also be considered. Additionally, the derivitized samples are stable for several hours. Multi user verification of the method (Figure 1) yielded a high level of reproducibility. The initial adsorption step takes place on a small quantity of the material, however, possibly limiting use to small volumes or previously concentrated samples. Considering the sensitivity this should not pose a problem and with the strong cation exchange SPE method developed here, sensitivity can be increased substantially but stability factors during concentration must also be considered.

No free or protein associated BMAA was detected in negative controls. Beta-N-methylamino-L-alanine was detected in 96% of freshwater isolates compared to the previously published 95% (Cox *et al.*, 2005). This difference is, however, statistically insignificant. Certain strains appear to have very low BMAA concentrations but it remains to be seen whether these low concentrations are a function of culture age, origin or culturing history. With increasingly sensitive detection methods the percentage of strains producing BMAA may increase. Given the ubiquity of the molecule it seems probable that all cyanobacteria produce BMAA to a greater or lesser extent. What is noteworthy is the lower concentration of both free and bound BMAA found in these novel isolates relative to previously published levels (Cox *et al*., 2005). An informal inter-laboratory test appeared to support the overestimation of BMAA by fluorescent detection of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatised samples compared to mass spectrometric detection of propyl chloroformate derivatised BMAA. However, given the variability in matrix correction and optimization of concentration in matrices prior to derivatization that is required, it is difficult to establish which of the methods reports a more accurate concentration. Irrespective of the accuracy of the quantification, what is clear is that the majority, if not all, cyanobacteria produce BMAA in sufficient quantities to pose a threat if that BMAA is in a form that can result in human or animal exposure. Furthermore, the presence of BMAA in a primary producer in aquatic ecosystems raises the question of possible bioaccumulation and biomagnification as occurs in the Guam ecosystem (Banack *et al*., 2006, 2007; Banack and Cox, 2003). This possibility increases the risk of human and animal exposure. Rao *et al.* (2006) and Lobner *et al*. (2007) showed that very low concentrations of BMAA are required to yield neurological damage and even motor neuron death. The extent of the risk to humans from direct exposure of free BMAA in these waters remains unknown, but may be significant, and the exposure via bioaccumulation and biomagnifcation may be even greater.

CONCLUSIONS

The LC-MS method presented here shows an adequate lower limit of detection and quantification and can be used for the rapid and reproducible detection and quantification of naturally occurring BMAA both in its free form within a complex organic matrix or in its protein associated form. The method is relatively simple and has the advantages of being standardized by the supplier and allowing the use of various analytical platforms. Additionally the method is extremely robust with regard multiple users and minor variations in use. Furthermore, the SPE method developed here allows the quantification of BMAA where the possibility of the molecule's presence in these waters due to bloom lysis or water treatment exists. Whether analyzing water or biomass hydrolysate, we recommend dilution to the lower limits of quantification for accurate quantification in a matrix so as to avoid matrix effects, particularly competition of SPE binding during derivatization.

Of the South African cyanobacteria tested, 96% contained BMAA. No taxonomic or geographic variation in BMAA content or ratio of free to protein associated BMAA was observed. Extraction from fresh samples yielded more BMAA but precludes quantification per dry weight unless adequate sample is obtained. We therefore recommend the adoption of this method for all analysis of BMAA in surface waters and cyanobacterial blooms or scums.

The increasing body of evidence linking BMAA to neurodegenerative diseases, and the potential for exposure to BMAA from cyanobacterial sources suggests the need for routine monitoring, the development and adoption of safety guidelines for BMAA, and further exposure and epidemiological studies.

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Appendix A – Research Outputs Resulting From Project

Journal Articles

BANACK SA, DOWNING TG, SPACIL Z, PURDIE EL, METCALF JS, DOWNING S, ESTERHUIZEN M, CODD GA, and COX PA (2010) Distinguishing the cyanobacterial neurotoxin β-N-methylamino-l-alanine (BMAA) from its structural isomer 2,4-diaminobutyric acid (2,4-DAB). Toxicon **56 (6)** 868-879.

ESTERHUIZEN M and DOWNING T (2008) β-N-methylamino-l-alanine (BMAA) in novel South African cyanobacterial isolates. Ecotoxicology and Environmental Safety **71 (2)** 309-313.

ESTERHUIZEN M, PFLUGMACHER S and DOWNING TG (2011) β-N-Methylamino-l-alanine (BMAA) uptake by the aquatic macrophyte Ceratophyllum demersum. Ecotoxicology and Environmental Safety **74 (1)** 74-77.

Conference Presentations

DOWNING TG. Cyanobacterial BMAA: Analysis, isomer differentiation and bioaccumulation. 6th Annual BMAA conference, Maimi, Florida, USA, November 2009 (Invited Speaker).

DOWNING TG. Cyanobacterial BMAA: South African research status and update. 5th Annual BMAA conference, Wyoming, USA, October 2008 (Invited Speaker).

ESTERHUIZEN M and DOWNING TG. β-methylamino-L-alanine (BMAA) in South African Cyanobacteria XII International Conference on Toxic Cyanobacteria, Rio de Janeiro State, Brazil, August 2007

ESTERHUIZEN M and DOWNING TG. Beta-N-methylamino-L-alanine induces oxidative stress in several aquatic organisms. 6th Annual BMAA conference, Maimi, Florida, USA, November 2009.

SEARLE S and DOWNING TG BMAA inhibits the growth of non-BMAA producing Synechocystis. 6th Annual BMAA conference, Miami, Florida, USA, November 2009

SEMBER CS, NEILAN BA, ESTERHUIZEN M and DOWNING TG. Cyanobacteria in the Eastern Cape, South Africa: Phylogeny and molecular screening of toxic isolates. XII International Conference on Toxic Cyanobacteria, Rio de Janeiro State, Brazil, August 2007

VISSER C, VAN DE VENTER M, KOEKEMOER T and DOWNING TG. Evaluation of model systems to study bioaccumulation of β-methylamino-l-alanine. XII International Conference on Toxic Cyanobacteria, Rio de Janeiro State, Brazil, August 2007