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 carbamatederivatized 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.