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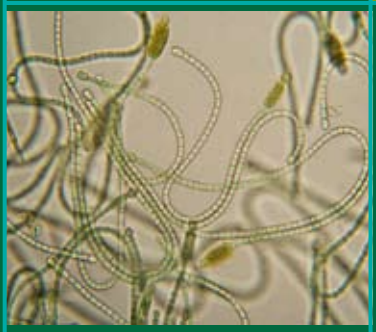
# BMAA in Potable Waters: an International Assessment of Exposure Levels



Research Report 65

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# BMAA in Potable Waters: An International Assessment of Exposure Levels

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## Research Report No 65

The Cooperative Research Centre for Water Quality and Treatment officially ended October 2008, and has been succeeded by Water Quality Research Australia Limited (WQRA), a company funded by the Australian water industry.



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BMAA in Potable Waters: An International Assessment of Exposure Levels
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# FOREWORD

Research Report Title: BMAA in Potable Waters: An International Assessment of Exposure Levels

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# EXECUTIVE SUMMARY

The primary aim of this project was to determine whether the potentially neurotoxic amino acid β-methylamino-L-alanine (BMAA) is produced by cyanobacteria that frequent Australian drinking water sources. In order to achieve this aim it was first necessary to establish high performance liquid chromatographic (HPLC) based analytical methods at project participant laboratories for the quantitative analysis of BMAA.

A literature-derived HPLC coupled to fluorescence (FL) detection method incorporating a novel extraction protocol was successfully established at the Australian Water Quality Centre for the analysis of BMAA in cyanobacteria. Analysis of ten natural and two cultured freshwater cyanobacteria samples originating from southern Australia tested negative for the presence of BMAA by HPLC-FL. These findings were also confirmed by state-of-the-art HPLC coupled to mass spectrometric (MS) analysis of replicate cyanobacteria extracts.

The results indicate that BMAA may not be an issue for the Australian water industry at the present time. However, these results differ from some published findings which detected BMAA in cyanobacterial samples from the UK and South Africa. Further studies are required to resolve these differences.

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# ABBREVIATIONS

| | |
|---|---|
| ALS/PDC | Amyotrophic Lateral Sclerosis/Parkinson's Dementia complex |
| AQC | 6-aminoquinolyl-N-hydroxysuccinimydyl carbamate |
| AWQC | Australian Water Quality Centre |
| BMAA | β-methylamino-L-alanine |
| BOAA | β-N-oxalylamino-L-alanine |
| EnTox | National Research Centre for Environmental Toxicology |
| HCl | Hydrochloric acid |
| HPLC-FL | High performance liquid chromatography coupled to fluorescence |
| HPLC-MS | High performance liquid chromatography coupled to mass spectrometry |
| LOD | Limit of detection |
| LOQ | Limit of quantitation |
| NL | Norleucine |
| RSD | Relative standard deviation |
| mg | Milligrams |
| μg | Micrograms |
| g | Grams |
| mM | Millimolar |
| μM | Micromolar |
| ppb | Parts per billion |
| ppm | Parts per million |

# 1 INTRODUCTION

## 1.1 Aim

The overall aim of this project was to determine whether BMAA is produced by cyanobacteria that frequent Australian drinking water sources. The project had the following objectives:

- Establishment of the appropriate high performance liquid chromatographic (HPLC) based analytical methods for the quantitative analysis of BMAA. This will include the establishment of appropriate sample preparation methods such as extraction and concentration of free and bound BMAA from cyanobacteria.

- Analysis of typical cyanobacteria found in South Australian freshwater supplies for the presence of bound and free BMAA.

## 1.2 Background

Algal blooms are being increasingly reported around the world in both marine and freshwater sources. They are known to produce chemicals that are hepatotoxic (microcystins and cylindrospermopsin) and neurotoxic (saxitoxins and anatoxins) toward animals and humans and therefore pose a significant problem to water utilities. Recently, another neurotoxin has been potentially associated with cyanobacteria, namely β-methylamino-L-alanine or BMAA (Fig. 1).

BMAA is associated with a fatal human neurodegenerative disease, with similarities to Alzheimer's and Parkinson's diseases. The disease (Amyotrophic Lateral Sclerosis/Parkinson's Dementia complex; ALS/PDC) was first described on Guam and BMAA was found to be produced by a symbiotic cyanobacterium (blue-green alga) living in specialized roots in cycad trees on the island (Cox *et al.*, 2003). BMAA has been found in the brain tissue of patients who died of ALS/PDC (Murch *et al.*, 2004a). It is concentrated in various parts of the cycad tree (Charlton *et al.*, 1992; Banack and Cox, 2003a) including the seeds which are used by the local people to produce flour. The flour is treated to remove toxins resulting in very little BMAA being ingested by this route. Consequently, flour was not considered to be a significant source of exposure to BMAA (Duncan *et al.*, 1990).

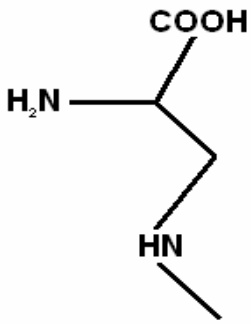



**Figure 1** Chemical structure of BMAA

It was recently hypothesised that, since flying foxes feed on cycad seeds and flying foxes are consumed by the people of Guam, this may be a route of exposure (Cox and Sacks, 2002). Subsequent analysis of flying foxes confirmed the accumulation of BMAA (Banack and Cox, 2003b). More recently, Cox *et al.* (2003) described the biomagnification of BMAA from the cyanobacterium, through the cycads, in the flying foxes which feed on the cycad seeds and in humans who eat the flying foxes. Thus a plausible route of significant exposure has been identified. This hypothesis is supported by the decline in ALS/PDC in recent years mirroring the decline in flying fox numbers (Monson *et al.*, 2003).

BMAA is found not only in its free form but also at higher levels bound in proteins, as are other normal protein amino acids, at all levels of the food chain (cyanobacteria, cycad plants including flour, flying

foxes and brain tissue). This suggests that these proteins function as an endogenous neurotoxic reservoir slowly releasing free toxin (Murch *et al.*, 2004b).

Neurodegenerative diseases in other areas of the Pacific have also been associated with exposure to cycad material (Spencer *et al.*, 1987a,b) which suggests that BMAA is involved in these disorders as well. A similar amino acid, β-N-oxalylamino-L-alanine (BOAA) is produced by the plant *Lathyrus sativus* and is responsible for a neurological disorder, neurolathyrism, when consumed (Olney, 1994).

Recent studies have shown that BMAA is also produced widely by free-living cyanobacteria from freshwaters throughout the world (Cox *et al.*, 2005). BMAA has also been found in brain tissue of not only people on Guam who had died of ALS/PDC but also Alzheimer's patients in Canada (Murch *et al.*, 2004a). Thus other sources of BMAA, possibly free-living cyanobacteria, may contribute to these types of neurological disorders (Cox *et al.*, 2003).

According to Cox *et al. (*2005) the BMAA-producing cyanobacteria include strains of the freshwater bloom-forming species *Cylindrospermopsis raciborskii* already of interest to the water industry. This suggests that the potential for human exposure to BMAA, via water, may be widespread.

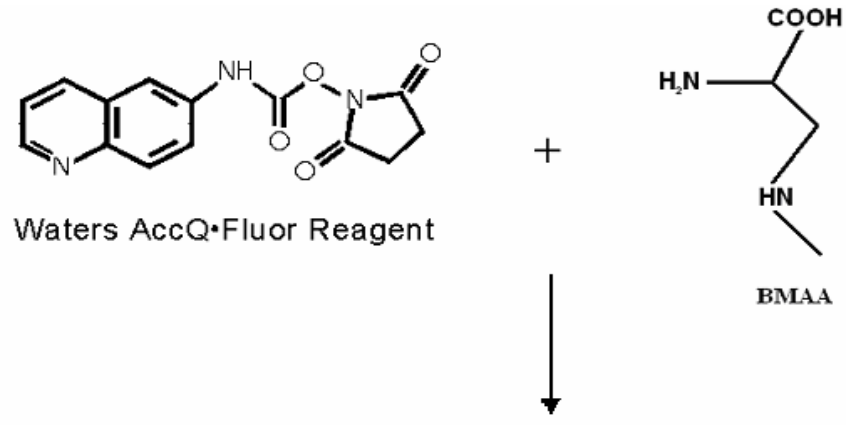
The detection of BMAA in a number of common cyanobacteria (Cox *et al.*, 2003) and the demonstrated capacity of BMAA to biomagnify raised concerns for the water industry. It was therefore decided to investigate the potential level of risk of exposure from drinking waters. Since BMAA was implicated as a potential cyanobacterial metabolite the research focus was on the screening of typical cyanobacteria that are found in drinking water sources in Australia and not drinking water itself.

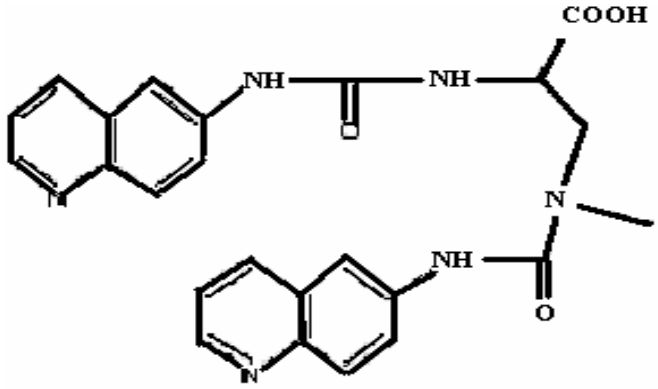
This project report describes results obtained from the following studies:

- Implementation and validation of a high performance liquid chromatographic method for BMAA at the Australian Water Quality Centre
- Determination of the extent of BMAA production by typical Australian cyanobacteria

## 1.3 Methods

The high performance liquid chromatography coupled to fluorescence (HPLC-FL) detection method reported by Cox *et al*. (2003) was chosen for BMAA analysis. Briefly, this method uses a commercially available reagent (Waters AccQ-Fluor) to chemically transform amino acids, including BMAA, to highly fluorescent and less polar urea derivatives known commercially as Waters AccQ amino acid derivatives (Figure 2).



**Waters AccQ derivative of BMAA**

**Figure 2** Chemical transformation of BMAA to Waters AccQ derivative

The BMAA AccQ derivative is then separated from other AccQ amino acid derivatives on a reverse phase HPLC column and subsequently detected by fluorescence.

A high performance liquid chromatography coupled to mass spectrometry or HPLC-MS method developed at EnTox, Queensland, Australia (not published) was chosen to confirm HPLC-FL analytical data obtained at the AWQC. For screening purposes cyanobacteria were analysed for total BMAA content and those found to contain BMAA were analysed for both free and bound BMAA. To determine total BMAA content suspensions of freeze-dried cyanobacteria were refluxed in 6 M hydrochloric acid for a period of 24 hours. This process extracted total BMAA.

# 2 EXPERIMENTAL

## 2.1 Equipment, chemicals and chromatographic materials

**Equipment**

Extracts were centrifuged using an Allegra™ 6 centrifuge (Beckman). Ultrafiltration of extracts was performed using a Hettich centrifuge model EBA 12. High performance liquid chromatography coupled to fluorescence (HPLC-FL) detection was performed using a Waters HPLC system comprising a 717 auto-sampler, 600 E multisolvent delivery system, 747 scanning fluorescence detector, temperature control module and Millennium 32 software.

**Chemicals and chromatography products**

BMAA and the internal standard nor-leucine (NL) were purchased from Sigma-Aldrich. Concentrated hydrochloric acid (HCL), triethylamine (analytical grade) and anhydrous sodium acetate (analytical grade) were purchased from Aldrich. High purity water was obtained from a Milli-Q water system. AccQ-Fluor reagent kit for the derivatisation of BMAA was purchased from Waters (includes derivatisation instructions). Ultrafree 0.5 Centrifugal filters (5,000 molecular weight (MW) cut-off) were purchased from Millipore. A Nova-Pak C-18 (300 mm x 4.6 mm internal diameter) high performance liquid chromatography (HPLC) column was purchased from Waters.

## 2.2 Cyanobacteria and cycad samples

The following natural and cultured (non-axenic) freeze-dried cyanobacteria cells and cycad sample were subjected to extraction and BMAA analysis in duplicate at the AWQC (Table 1).

**Table 1.** Cyanobacteria and cycad samples extracted and analysed for BMAA

| Cyanobacteria | Sample location, year | Amount extracted in (mg) for analysis of total BMAA content | |
|---|---|---|---|
| | | **Replicate #1** | **Replicate #2** |
| *Nodularia* (A) | Victoria, 1996 | 197.2 | 197.3 |
| *Microcystis flos aquae* (B) | South Australia, 2002 | 120.3 | 124.6 |
| *Cylindrospermopsis raciborskii* (C) | AWQC Culture #1, 2007 | 90.0 | 75.0 |
| *Cylindrospermopsis raciborskii* (D) | AWQC culture #2, 2007 | 110.1 | 100.8 |
| *Microcystis aeruginosa* (E) | Unknown location | 116.7 | 110.7 |
| *Anabaena circinalis* (F) | South Australia, site #1 | 107.4 | 106.6 |
| *Anabaena circinalis* (G) | South Australia, site #2 | 120 | 114 |
| *Planktolyngbya algae* (H) | Western Australia | 100 | 99.2 |
| *Phormidium tychomena* (I) | Barossa Valley, South Australia | 103.5 | 102 |
| *Microcystis wesenbergi* (J) | New South Wales | 111 | 98.5 |
| *Benthic oscillatoria* (K) | South Australia | 110 | - |
| *Microcystis flos aquae* (L) | Torrens Lake, South Australia | 102.5 | 101 |
| **Cycad** | **Sample location, year** | **Amount extracted in (mg)** | |
| *Cycas rumphi** | Brisbane, Queensland, 2007 | 102 / 106 (for total BMMA content)<br>109.5 / 111.8 (for free BMAA content) | |

## 2.3 Solutions

**Preparation of ~20 mM HCL**

1 mL of concentrated HCL (~10 M) was diluted to a final volume of 0.5 L with Milli-Q water. This solution was kept in a sealed inert screw cap glass bottle (Shot bottle) prior to use.

**Preparation of ~ 6 M HCl**

60 mL of concentrated HCL (~10 M) was diluted to a final volume of 100 mL with Milli-Q water. This solution was kept in a sealed inert screw cap glass bottle prior to use.

**Preparation of 6.49 mM or 1000 ppm Stock BMAA Solution (S-BMAA)**

To BMAA (2.0 mg, MW ~154) was added 20 mM HCL (2 mL). The solution was prepared in an amber coloured glass screw capped HPLC vial. Gentle swirling was required to dissolve the BMAA. The solution was stored at $4^0$C prior to use.

**Preparation of 1 mM or 131 ppm Norleucine (NL) Internal Standard Stock Solution for calibration solutions (S-NL-CAL)**

To D-norleucine (2.62 mg , MW ~ 131) was added 20 mM HCL (20 mL). The solution was prepared in an amber coloured glass screw capped HPLC vial. Gentle swirling was required to dissolve the NL. The solution was stored at $4^0$C prior to use.

**Preparation of 10 mM or 1310 ppm Norleucine (NL) Internal Standard Stock Solution for extracts (S-NL-EX)**

To D-norleucine (2.62 mg, MW ~ 131) was added 20 mM HCL (2 mL). The solution was prepared in an amber coloured glass screw capped HPLC vial. Gentle swirling was required to dissolve the NL. The solution was stored at $4^0$C prior to use.

**Preparation of BMAA Calibration Standards for HPLC-FL Analysis Containing NL as Internal Standard**

Calibration standards (CAL-1-4) were prepared from dilution of S-BMAA and S-NL as described in Table 2 *(stock BMAA and NL was brought to room temperature before being used for preparation of the calibrations standards).*

**Table 2.** Preparation of BMAA calibration stock solutions

| BMAA Calibration standard stocks | S-BMAA (µL) | S-NL-CAL (µL) | Milli-Q water (µL) | Concentration of BMAA in calibration stocks (mM/ppm) |
|---|---|---|---|---|
| #1 | 0 | 80 | 420 | 0 |
| #2 | 100 | 80 | 320 | 1.3 / 200 |
| #3 | 200 | 80 | 220 | 2.6 / 400 |
| #4 | 300 | 80 | 120 | 3.89 / 600 |

*All contain 160 µM or 20.96 ppm NL; All calibration solutions should be divided in to 100 µL aliquots and stored at -$20^0$C prior to use.

## 2.4 Derivatisation of calibration standards

The calibration stock solutions shown above in table 2 were derivatised as per the Waters AccQ-Fluor method provided with the derivatisation Kit. The Waters AccQ fluor Reagent Kit contained AccQ Fluor Borate pH 8.8 buffer (vial 1), AccQ Fluor Reagent Powder (vial 2A) which contained the derivatisation reagent 6-aminoquinolyl-N-hydroxysuccinimydyl carbamate (AQC) and AccQ Fluor solvent (acetonitrile) (vial 2B). The derivatisation instructions provided with the kit were as follows:

### 2.4.1 Preparation of the derivatisation reagent

- Preheat a heating block to 55°C
- Tap vial 2A lightly before opening to ensure all AccQ Fluor Reagent powder is at the bottom of the vial.
- Rinse a clean micropipette by drawing and discarding 1 mL of AccQ Fluor Reagent solvent from vial 2B
- Transfer 1 mL of AccQ Fluor Reagent solvent to the AccQ Fluor Reagent powder in vial 2A
- Cap vial 2A lightly and vortex for 10 seconds
- Heat vial 2A on the top of the heating block, vortex occasionally, until the powder dissolves

### 2.4.2 Derivatisation procedure

- Preheat a heating block to 55°C
- Use a clean syringe to deliver 10 µL of calibration standard to the bottom of a clean 6 x 50 mm sample tube
- Use a micropipette to add 70 µL of AccQ Fluor Borate Buffer (vial 1) to the sample tube. Vortex briefly
- Use a micropipette with a clean tip to add 20 µL of reconstituted AccQ Fluor Reagent to the 6 x 50 mm sample tube. Vortex immediately for several seconds
- Allow to incubate for 1 minute at room temperature
- Use a 9-Inch Pasteur pipette to transfer the contents of the sample tube to the bottom of an autosample vial limited volume insert. Cap with a silicone-lined septum
- Heat the vial in a heating block for 10 minutes at 55°C

The reagent quantities used to derivatise the calibration standards are summarised in Table 3 below:

**Table 3.** Derivatisation of calibration solutions

| Concentration of final BMAA in derivatisation sol. mM /ppm* | Initial BMAA calibration stocks (µL) | AccQ.Fluor Borate (0.2 M sodium borate at pH 8.8) Buffer (µL) | AccQ.Fluor Reagent (µL)** |
|---|---|---|---|
| 0 / 0 (Cal-1) | #1 / 10 | 70 | 20 |
| 0.13 / 20 (Cal-2) | #2 / 10 | 70 | 20 |
| 0.26 / 40 (Cal-3) | #3 / 10 | 70 | 20 |
| 0.389 / 60 (Cal-4) | #4 / 10 | 70 | 20 |

*The concentration of derivatives internal standard (NL) in the final solutions is 16 µM / 2.1 ppm assuming the derivatisation reaction proceeded quantitatively. **The concentration of the derivatisation reagent in the reaction mixtures is 2000 µM or 570 ppm

## 2.5 Preparation and derivatisation of limit of detection and quantitation samples

Limit of detection (LOD) and limit of quantitation (LOQ) solutions were prepared as shown in Table 4 below. LOD / LOQ solutions #1-4 were derivatised as described above for the calibration standards.

**Table 4.** Preparation of LOD and LOQ solutions

| LOD / LOQ solutions | Cal-2 stock (µL) | Milli-Q water (µL) | BMAA concentration in LOD/LOQ solutions (mM / ppm) |
|---|---|---|---|
| #1 | 50 | 100 | 0.43 / 66.7 |
| #2 | 50 | 200 | 0.26 / 40 |
| #3 | 50 | 450 | 0.13 / 20 |
| #4 | 50 | 950 | 0.065 / 10 |

## 2.6 Extraction and derivatisation of cyanobacteria and cycad samples

**Extraction and derivatisation of total BMAA from cyanobacteria or cycad with internal standard added following extraction***

To freeze-dried cyanobacteria or cycad (see Table 1 in Section 2.2) was added 6 M HCl (10 mL). The resultant suspension was left to stir at reflux (110$^0$C) for 24 hours. The suspension was then cooled and centrifuged to remove particulate cellular material. The pellet was then re-suspended in Milli-Q water (10 mL) and centrifuged (4,000 x g) in order to extract residual BMAA. The collected supernatants were combined and concentrated to dryness using a rotary evaporator under reduced pressure. The residue was dissolved in Milli-Q water (5 mL) and freeze-dried. The freeze-dried material was then dissolved in a mixture of Milli-Q water (1680 µL) and S-NL-EX (320 µL). A 500 µL aliquot (remaining 1.5 mL was stored at -20$^0$C) was then ultrafiltered using an ultra-free 0.5 KD centrifugal filter (5,000 mwt cut-off). The ultra-filtrate was then diluted 1:10 with Milli-Q water. An aliquot (10 µL) of this solution was then derivatised as described for the calibration standards except in this case volumes of derivatisation solvent and reagent used were 60 µL and 30 µL respectively.

**Nodularia* (A) was also extracted without NL addition.

**Extraction and derivatisation of BMAA from cyanobacteria with internal standard added prior to extraction**

To freeze-dried cyanobacteria (see Table 1 in Section 2.2) was added 6 M HCl (10 mL) followed by S-NL-EX (320 µL). The resultant suspension was left to stir at reflux (110$^0$C) for 24 hours. The suspension was then cooled and centrifuged to remove particulate cellular material. The pellet was then re-suspended in Milli-Q water (10 mL) and centrifuged (4,000 x g) in order to extract residual BMAA. The collected supernatants were combined and concentrated to dryness using a rotary evaporator under reduced pressure. The residue was dissolved in Milli-Q water (5 mL) and freeze-dried. The freeze-dried material was then dissolved in a mixture of Milli-Q water (2 mL). The solution was then ultrafiltered and derivatised as described above (see Extraction and derivatisation of BMAA from cyanobacteria with internal standard added following extraction).

**Extraction and derivatisation of free BMAA from a cycad sample**

To the cycad sample (see Table 1 in Section 2.2) was added Milli-Q water (4.2 mL) followed by S-NL-EX (800 µL). The suspension was sonicated for 10 min and then centrifuged @ 3,000 x g for 10 min. A 0.5 mL aliquot of the supernatant was then ultrafiltered and derivatised as described above (see Extraction and derivatisation of BMAA from cyanobacteria with internal standard added following extraction).

## 2.7 HPLC-FL analysis

**Mobile phase preparation**

Eluent A (140 mM sodium acetate, 5.6 mM triethylamine, pH 5.2): Sodium acetate (11.48 g) and triethylamine (0.565 g) were dissolved in Milli-Q water (500 mL) and then this solution was further diluted with Milli-Q water to give a final volume of 1 L. The pH of this solution was then adjusted to 5.2 by addition (drop by drop) of concentrated phosphoric acid.

Eluent B (60% (v/v) acetonitrile in water): To Milli-Q water (400 ml) was added acetonitrile to give a final volume of 1 L.

**Analysis**

Derivatised calibration standards and sample extracts (5 µL) were injected on to a Waters Nova-Pak $C_{18}$ column, 300 x 3.9 mm thermostatted @ $37^0$C and separated using the gradient described in Table 5. Eluted amino acid AccFlu derivatives were detected by fluorescence (excitation @ 250 nm and emission @ 395 nm). Calibration standards and derivatised extracts were generally analysed following the sample set arrangement shown in Table 6.

**Table 5.** HPLC gradient conditions

| Time (min) | Gradient (flow rate:1 mL/min) |
|---|---|
| 0-2 | 100% A to 90% A (curve 11) |
| 2-5 | 90% A to 86% A (curve 11) |
| 5-10 | 86% A (curve 6) |
| 10-18 | 86% A to 73% A (curve 6) |
| 18-30 | 73% A to 60% A (curve 10) |
| 30-35 | 60% A to 40% A (curve 6) |
| 35-39 | 40% A to 10% A (curve 6) |
| 39-40 | 10% A to 100% B (curve 6) |
| 40-45 | 100% B to 100 %A (curve 6) |
| 45-50 | Equilibration at 100% A prior to next injection |

**Table 6.** General sample set running protocol for a group of five samples

| Sample | Volume (µL) and number of injections | Comments |
|---|---|---|
| Calib std #1 | 5/2 | A four point calibration curve (includes zero concentration and must cover the BMAA concentration range anticipated in the sample) should be constructed using the mean of the peak area responses obtained from duplicate BMAA standard injections versus concentrations. |
| Calib std #2 | 5/2 | |
| Calib std #3 | 5/2 | |
| Calib std #4 | 5/2 | |
| Sample #1 | 5/2 | Mean peak areas obtained from duplicate injections should be used to determine concentration from the calibration curve. |
| Sample #2 | 5/2 | |
| Sample #3 | 5/2 | |
| Sample #4 | 5/2 | |
| Sample #5 | 5/2 | |
| Check std | 5/2 | Duplicate injections of one of the four calibration standards should be conducted and the mean peak area response obtained should be compared to the initial mean used to construct the calibration curve. |

# 3 RESULTS

## 3.1 Establishment of HPLC-FL (Cox *et al*. 2003) at the AWQC for the analysis of BMAA in cyanobacteria

Prior to utilising the HPLC-FL method reported by Cox *et al.* (2003) at the AWQC for the screening of cyanobacteria for the presence of BMAA it was essential to establish the basic precision elements of the method. Therefore, sections 3.1.1 - 3.1.3 address the reproducibility of BMAA and NL peak retention time and area, linearity of detector and limit of BMAA detection (LOD) and quantitation (LOQ).

### 3.1.1 Reproducibility of peak retention time and area

Figure 3 shows a representative chromatogram of Cal-3 containing BMAA and the internal standard norleucine (NL) in concentrations of 60 and 2.1 ppm, respectively. Figures 4 and 5 show graphical representations of the BMAA peak retention times and areas observed for seven consecutive replicate analyses of Cal-3. The mean peak retention time of BMAA was found to be 31.56 min with a relative standard deviation (RSD) of 0.65% as shown in Table 6. The RSD of the peak area for BMAA over seven injections was 8.17%. The mean retention time and RSD for NL was 37.87 min and 0.13% respectively (Table 7).

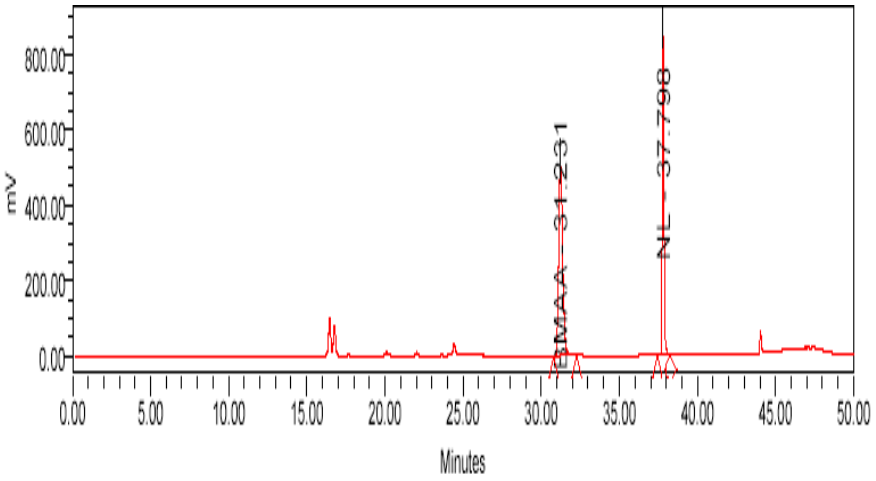



**Figure 3.** A replicate HPLC-FL chromatogram of Cal-3

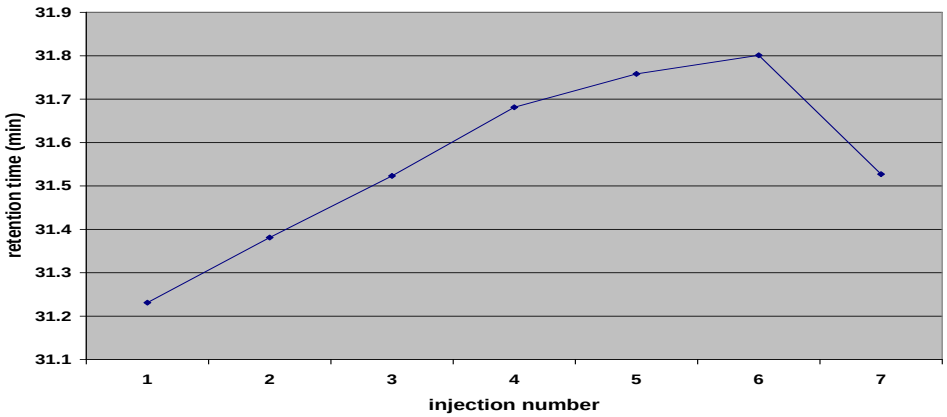



**Figure 4.** Graphical representation of BMAA peak retention time for seven replicate HPLC-FL analyses of Cal-3


**Figure 5.** Graphical representation of BMAA peak areas for seven replicate HPLC-FL analyses of Cal-3.

**Table 7.** Peak areas, peak retention times and relative standard deviations for BMAA and NL following seven replicate HPLC-FL analyses of Cal-4

| Injection | BMAA peak | | Norleucine peak | |
|---|---|---|---|---|
| | Retention time | Area | Retention time | Area |
| 1 | 31.231 | 9208943 | 37.798 | 6573125 |
| 2 | 31.381 | 9785416 | 37.849 | 6767856 |
| 3 | 31.523 | 9726815 | 37.871 | 7299757 |
| 4 | 31.681 | 7716550 | 37.9 | 6081636 |
| 5 | 31.758 | 9643099 | 37.925 | 7306619 |
| 6 | 31.801 | 9010576 | 37.907 | 6870444 |
| 7 | 31.527 | 9862685 | 37.805 | 7481705 |
| **Mean** | **31.56** | **9279155** | **37.865** | **6911592** |
| **RSD (%)** | **0.65** | **8.17** | **0.13** | **7.13** |

## 3.1.2 Linearity of detector response

Four Milli-Q water solutions each containing AccQ derivatised internal standard (NL) in a concentration of 2.1 pm and AccQ derivatised BMAA in concentrations of 0 (Cal-1), 20 ppm (Cal-2), 40 ppm (Cal-3) and 60 ppm (Cal-4) respectively, were analysed in duplicate by HPLC-FL (see section 2.6). The mean BMAA peak areas obtained for each calibration standard together with the corresponding NL internal standard response (Figure 6) were subjected to linear regression analysis using Waters HPLC Millenium software. The subsequent BMAA calibration curve (Figure 7) yielded an $R^2$ value of 0.998115.

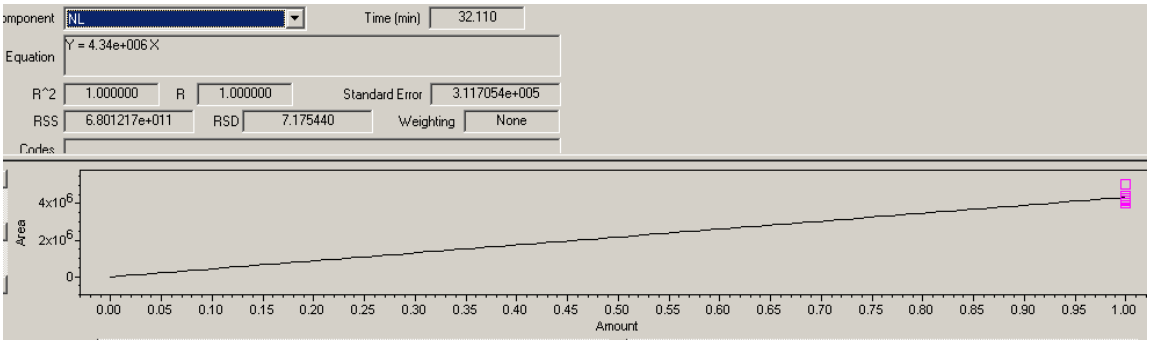



**Figure 6.** Peak area response of NL internal standard

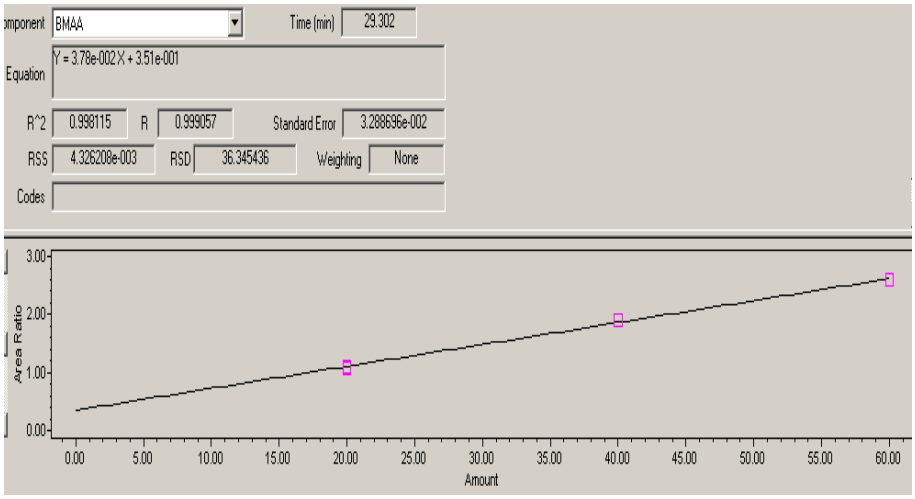



**Figure 7.** BMAA calibration curve

## 3.1.3 Limit of BMAA detection and quantitation

Four duplicate Milli-Q water solutions containing AccQ derivatised BMAA in concentrations ranging from 1 to 6.7 ppm (see above in Table 4) were analysed by HPLC-FL (Figures 8-11) in order to extrapolate approximate limit of BMAA detection (LOD) and quantitation (LOQ) values.

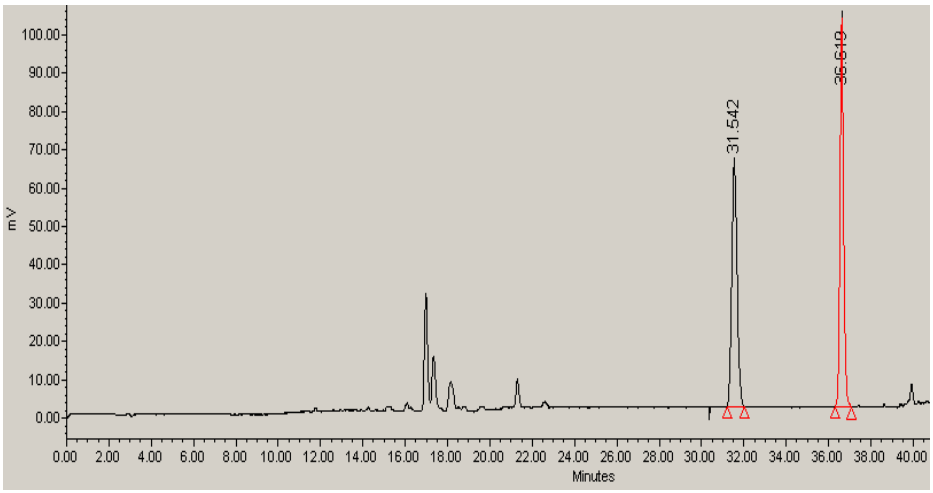



**Figure 8.** Replicate HPLC-FL chromatogram of a Milli-Q water solution containing AccQ derivatised BMAA and NL in concentrations of 6.7 and 0.7 ppm respectively

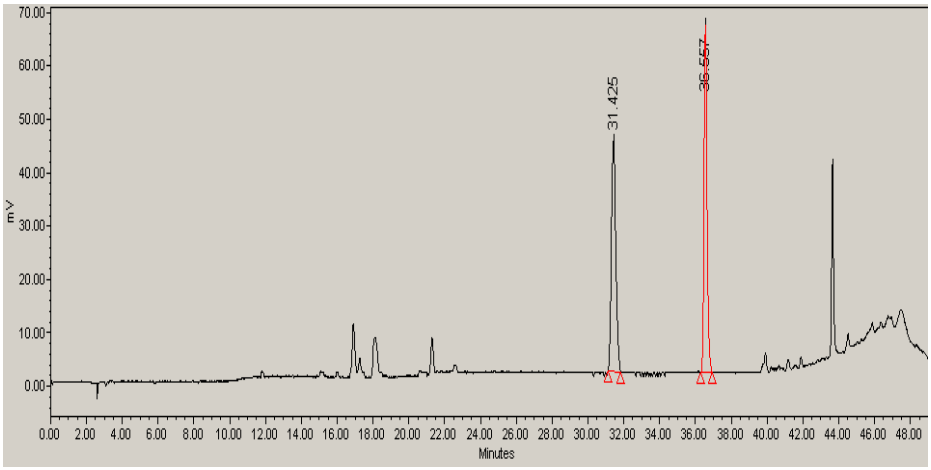



**Figure 9.** A replicate HPLC-FL chromatogram of a Milli-Q water solution containing AccQ derivatised BMAA and NL in concentrations of 4.0 and 0.42 ppm respectively

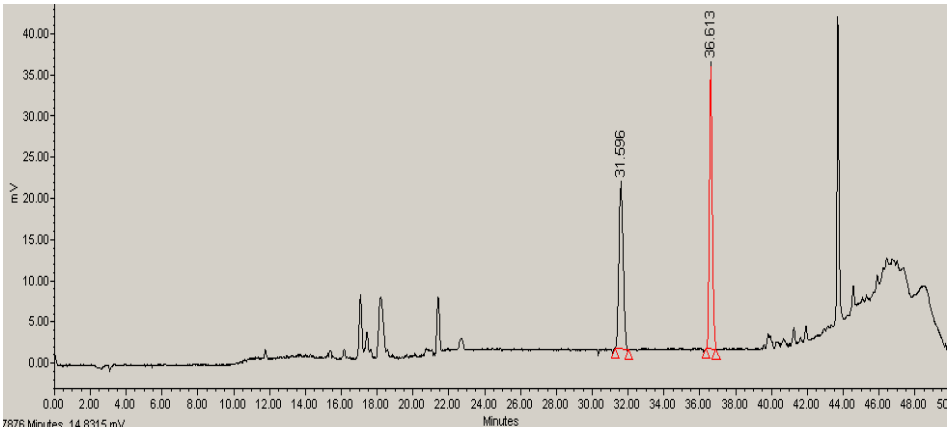



**Figure 10.** A replicate HPLC-FL chromatogram of a Milli-Q water solution containing AccQ derivatised BMAA and NL in concentrations of 2.0 and 0.21 ppm respectively

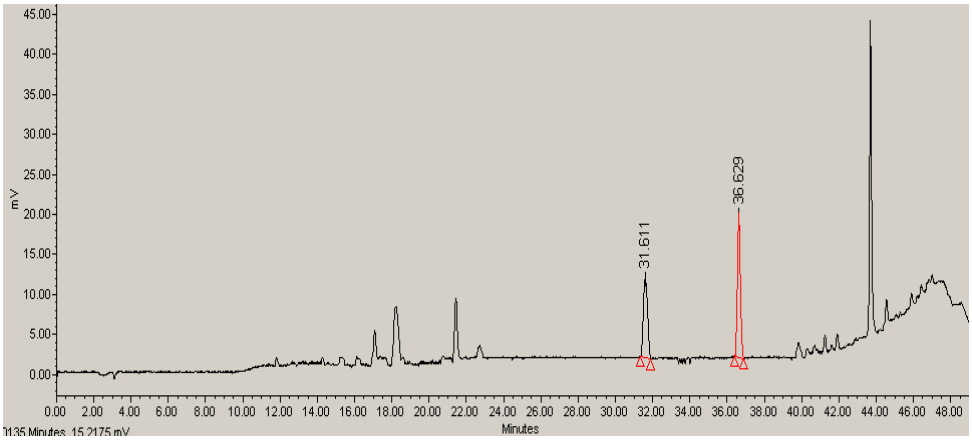



**Figure 11.** A replicate HPLC-FL chromatogram of a Milli-Q water solution containing AccQ derivatised BMAA and NL in concentrations of 1.0 and 0.11 ppm respectively

The approximate noise level determined by magnifying the baseline (zoom not shown) and measuring the height of a typical background noise peak approximately 1 min left from the BMAA peak in all of the replicate chromatograms (Figures 8-11) was 0.2 millivolts (mV). The average BMAA

peak height of the duplicate chromatograms for each solution was converted to BMAA peak height (x) to noise ratio (x : 1) values. Figure 10 shows a plot of BMAA signal to noise ratio versus BMAA concentration for each solution. Linear regression analysis of the plot gave an $R^2$ value of 0.9896 indicating good detector linearity over this BMAA concentration range. Approximate BMAA LOD and LOQ concentrations of 0.1 and 0.2 ppm representing signal to noise ratios of 5 : 1 and 10 : 1 respectively were extrapolated from the plot (Fig. 12).

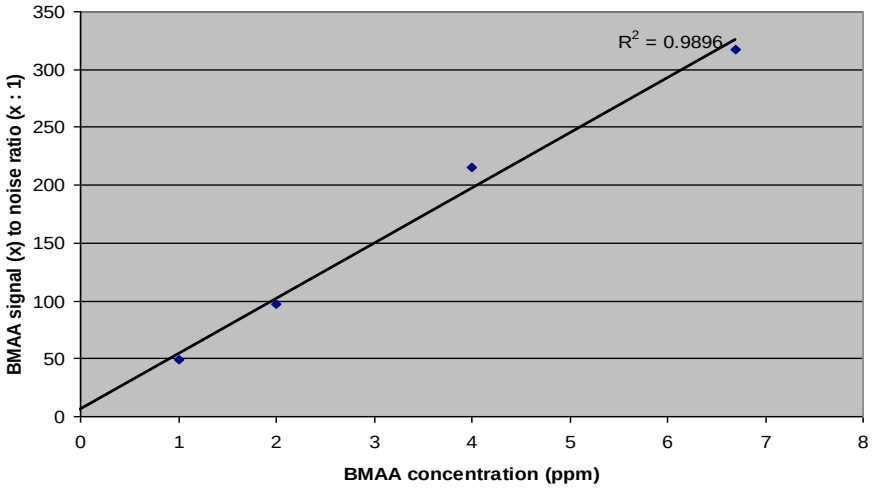



**Figure 12.** BMAA signal to noise ratio for HPLC-FL analysis

Unfortunately due to project time constraints the LOD and LOQ extrapolated values could not be verified by analysing solutions of BMAA present in these concentrations.

## 3.2 Analysis of cyanobacteria extracts for the presence of BMAA

Following the establishment of the basic precision elements of the HPLC-FL method as described above in section 3.1, it was necessary to establish the effects of cyanobacteria matrix impurities on the recovery of BMAA and also to verify that the HPLC-FL method can detect BMAA present in a natural BMAA containing biological reference sample. Section 3.2.1 shows the results of HPLC-FL analysis of a cycad sample (*Cycas rumphii*, Brisbane, Queensland, 2008) containing total and free BMAA (see section 2.6 for details of extraction methods). Section 3.2.2 gives the BMAA recovery results obtained for the cyanobacterial extraction process and the subsequent BMAA screening results for a selection of natural and cultured Australian cyanobacteria samples respectively.

## 3.2.1 Analysis of a cycad reference sample

Figures 13 and 14 represent replicate HPLC-FL chromatograms of a cycad extract showing its total and free BMAA content as evidenced by the peaks at 29.58 min and 29.55 min respectively. The peaks for the internal standard or IS (NL) were present at 32.32 (Fig. 13) and 32.29 (Fig 14) min.

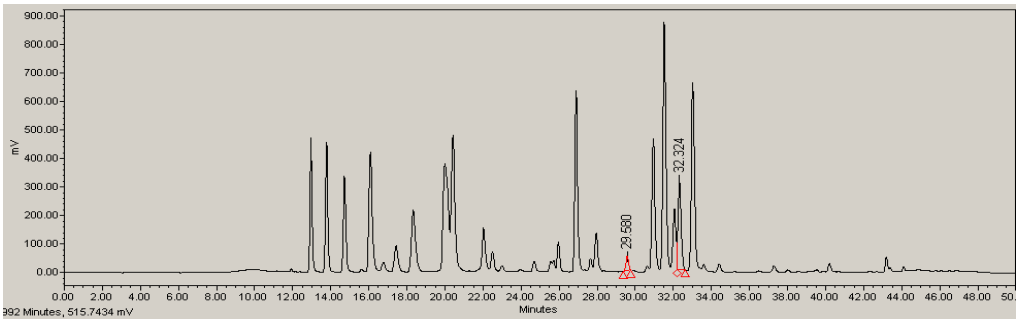



**Figure 13.** HPLC-FL chromatogram of a cycad extract showing total BMAA content

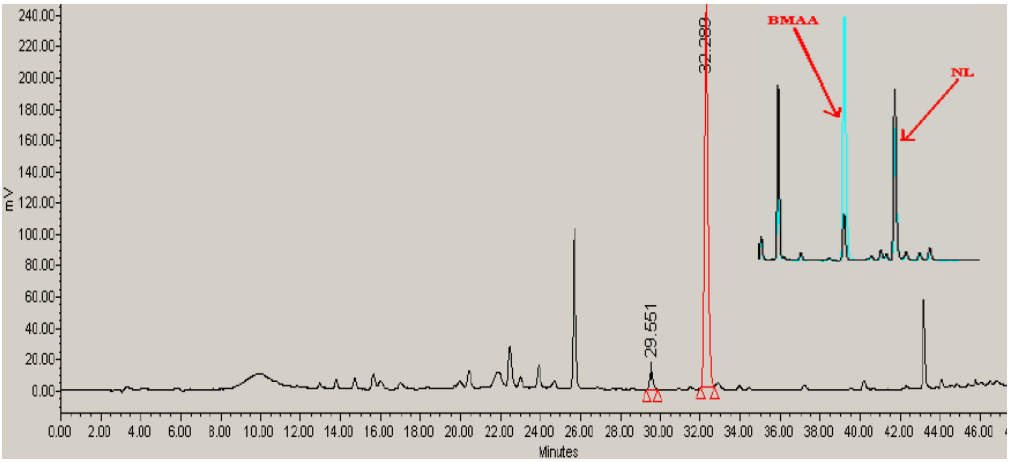


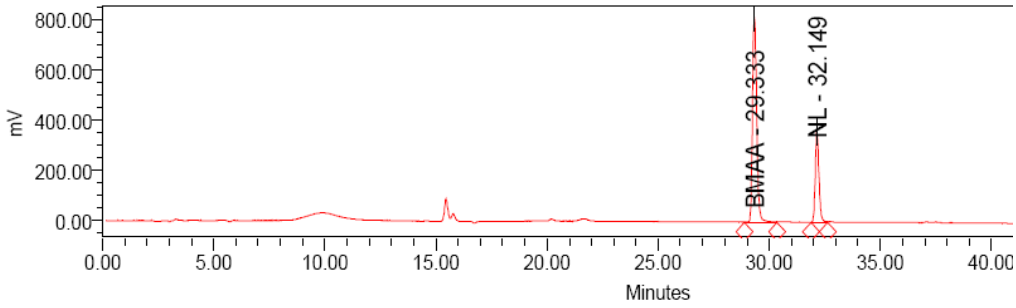

**Figure 14.** HPLC-FL chromatogram of a cycad extract showing free BMAA content (insert shows non BMAA and BMAA spiked extract)

The mean recovery (from duplicate analysis) of NL from the total BMAA and free BMAA extraction process was 65.15% and 52% respectively, determined from comparison of the mean NL peak areas of the calibration standards with those from the samples (calibration not shown). On a BMAA weight per cycad weight basis, the mean concentration value calculated for total and free BMAA content was 509.6 µg/g and 433.8 µg/g respectively. By subtraction of the free value from the total BMAA value the bound BMAA content in the cycad sample was 75.8 µg/g indicating that the cycad sample contained BMAA mostly in the free amino acid form.

## 3.2.2 Screening of Australian cyanobacteria for the presence of BMAA

As indicated previously, to simplify the screening of cyanobacteria for the presence of BMAA we decided to treat cyanobacteria samples with strong acid to extract free and/or bound BMAA and also to hydrolyse bound BMAA (if present) to the free amino acid form in one step. In order to gauge where BMAA loss would be most significant during the entire analytical method (five steps: extraction of BMAA, hydrolysis of bound BMAA, ultrafiltration, derivatisation of BMAA with AccQ fluor tag, HPLC-FL analysis) a cyanobacteria sample (A) was spiked with NL following the hydrolysis stage and a replicate sample not spiked with NL. Both extracts were then derivatised and analysed by HPLC-FL. The remainder of the cyanobacteria samples were spiked with NL at the very beginning of the analytical process (see section 2.6 for details) and screened for the presence of BMAA.

Figure 15 shows a HPLC-FL chromatogram of one of the calibration standards (Cal-4) run immediately prior to the analysis of the cyanobacteria extracts. The mean HPLC-FL retention times of BMAA and NL for the four calibration standards analysed were 29.34 and 32.15 min respectively.

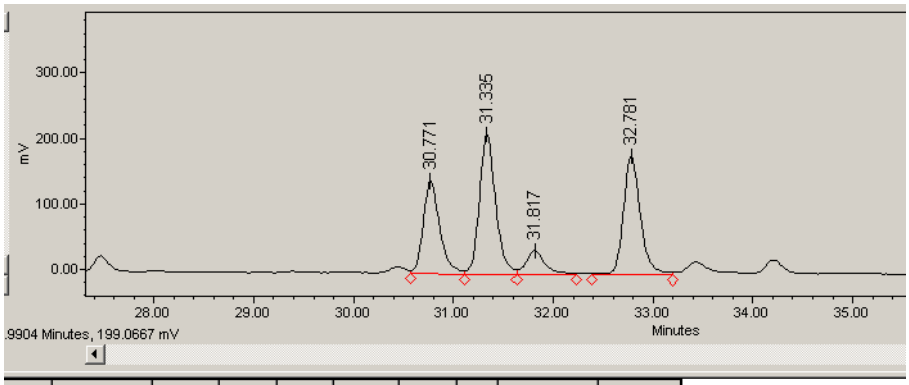



**Peak Results**

| | SampleName | Name | Sample Type | RT | Area | Amount | Units |
|---|---|---|---|---|---|---|---|
| 1 | cal 4 | BMAA | Standard | 29.333 | 10846829 | 60.000 | ppm |
| 2 | cal 4 | NL | Standard | 32.149 | 4163825 | 1.000 | ppm |

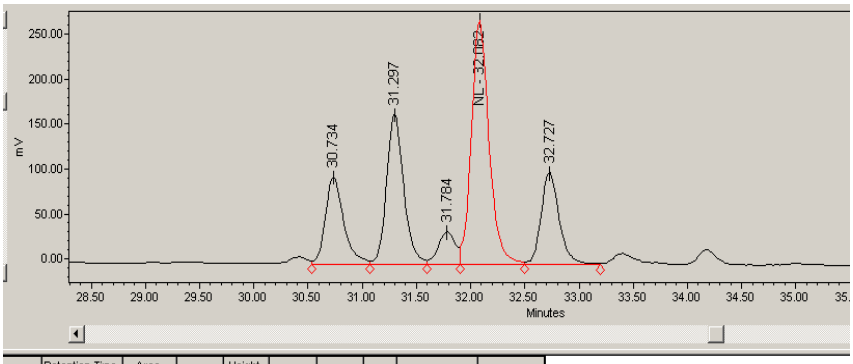
**Figure 15.** HPLC-FL chromatogram of Cal-4

Figures 16 and 17 show HPLC-FL chromatograms of extracts of *Nodularia* (A) representing no IS spike and IS spiked at a final NL concentration of 2.1 ppm (see section 2.7). Figure 16 indicates that the *Nodularia* extract contained no BMAA and NL as evidenced by a lack of peaks at approximately 29.3 and 32.15 minutes respectively. Figure 17 shows the NL spike as evidenced by the peak at approximately 31.1 min. By comparison of the mean peak area of NL from the four calibration standards (only Cal-4 is shown in Fig 15) to that of the NL peak in figure 17, NL recovery was found to be 73.1%.




| Name | Retention Time (min) | Area (µV*sec) | % Area | Height (µV) | Int Type | Amount | Units | Peak Type | Peak Codes |
|---|---|---|---|---|---|---|---|---|---|
| BMAA | 29.349 | | | | Missing | | | Missing | |
| | 30.771 | 1646561 | 24.64 | 142919 | VV | | | Unknown | |
| | 31.335 | 2392195 | 35.79 | 214213 | VV | | | Unknown | |
| | 31.817 | 480667 | 7.19 | 36045 | VV | | | Unknown | |
| NL | 32.147 | | | | Missing | | | Missing | |

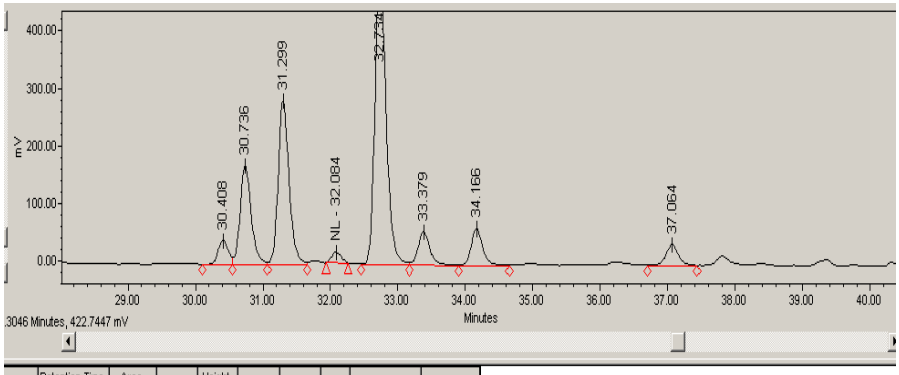
**Figure 16** Zoomed HPLC-FL chromatogram of a natural *Nodularia* extract containing no NL




| Name | Retention Time (min) | Area (µV*sec) | % Area | Height (µV) | Int Type | Amount | Units | Peak Type | Peak Codes |
|---|---|---|---|---|---|---|---|---|---|
| BMAA | 29.349 | | | | Missing | | | Missing | |
| | 30.734 | 1134669 | 14.76 | 97351 | VV | | | Unknown | |
| | 31.297 | 1855597 | 24.14 | 167403 | VV | | | Unknown | |
| | 31.784 | 393679 | 5.12 | 36109 | VV | | | Unknown | |
| NL | 32.082 | 3104885 | 40.40 | 271138 | VV | 1.000 | | Int Std | |

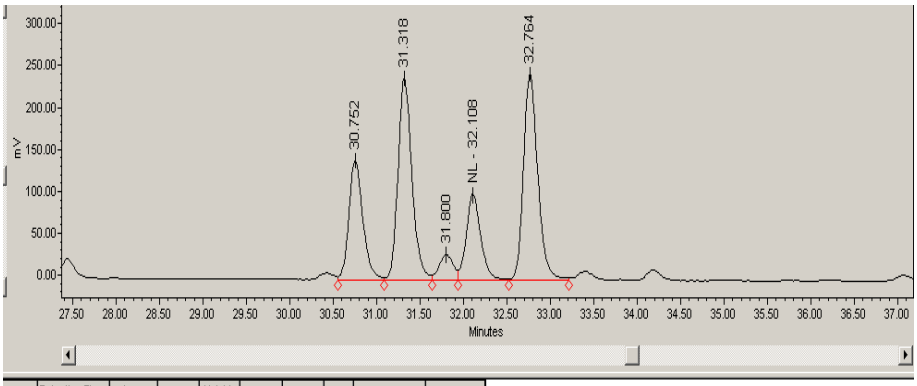
**Figure 17.** Zoomed HPLC-FL chromatogram of a natural *Nodularia* extract spiked with NL following hydrolysis

Figures 18-20 represent HPLC-FL chromatograms of cyanobacteria extracts B, D and E. HPLC-FL profiles of all the samples tested were similar with respect to each other. Interestingly and in contrast to the NL recovery obtained above for the *Nodularia* extract (A), the mean NL recovery for samples B to L was 20% indicating that the internal standard degrades substantially during the hydrolysis stage of the analytical method. No BMAA was detected by HPLC-FL in any of the twelve cyanobacteria samples tested during this project (Table 8). This finding was confirmed by HPLC-FLMS analysis of the non-spiked underivatised extracts (results not shown: performed by Dr Wasa Wickramsinghe, NRCET, Brisbane, Queensland).



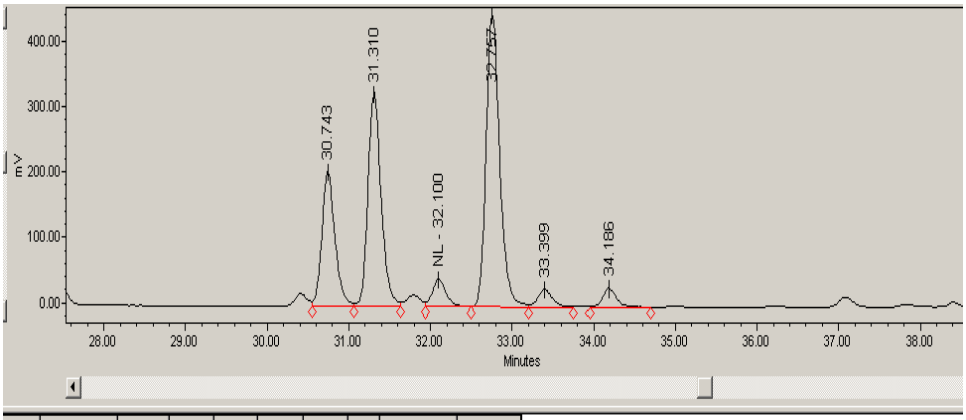

| Name | Retention Time (min) | Area (µV*sec) | % Area | Height (µV) | Int Type | Amount | Units | Peak Type | Peak Codes |
|---|---|---|---|---|---|---|---|---|---|
| BMAA | 29.349 | | | | Missing | | | Missing | |
| | 30.408 | 444980 | 3.22 | 41622 | VV | | | Unknown | |
| | 30.736 | 1977950 | 14.31 | 173406 | VV | | | Unknown | |
| | 31.299 | 3216718 | 23.27 | 286016 | VV | | | Unknown | |
| NL | 32.084 | 175341 | 1.27 | 18355 | bb | 1.000 | | Int Std | |

**Figure 18.** HPLC-FL profile of an extract from cultured *Microcystis flos aquae* (B)




| Name | Retention Time (min) | Area (µV*sec) | % Area | Height (µV) | Int Type | Amount | Units | Peak Type | Peak Codes |
|---|---|---|---|---|---|---|---|---|---|
| BMAA | 29.349 | | | | Missing | | | Missing | |
| | 30.752 | 1586626 | 18.58 | 143214 | VV | | | Unknown | |
| | 31.318 | 2664187 | 31.21 | 241268 | VV | | | Unknown | |
| | 31.800 | 336188 | 3.94 | 30366 | VV | | | Unknown | |
| NL | 32.108 | 1165320 | 13.65 | 101910 | VV | 1.000 | | Int Std | |

**Figure 19.** HPLC-FL profile of an extract from a cultured *Cylindrospermopsis raciborskii* (D)




| Name | Retention Time (min) | Area (µV*sec) | % Area | Height (µV) | Int Type | Amount | Units | Peak Type | Peak Codes |
|---|---|---|---|---|---|---|---|---|---|
| BMAA | 29.349 | | | | Missing | | | Missing | |
| | 30.743 | 2316051 | 18.80 | 207184 | VV | | | Unknown | |
| | 31.310 | 3650833 | 29.63 | 327099 | VV | | | Unknown | |
| NL | 32.100 | 499747 | 4.06 | 41847 | VV | 1.000 | | Int Std | |

**Figure 20.** HPLC-FL profile of an extract from a natural *Microcystis aeruginosa* (E)

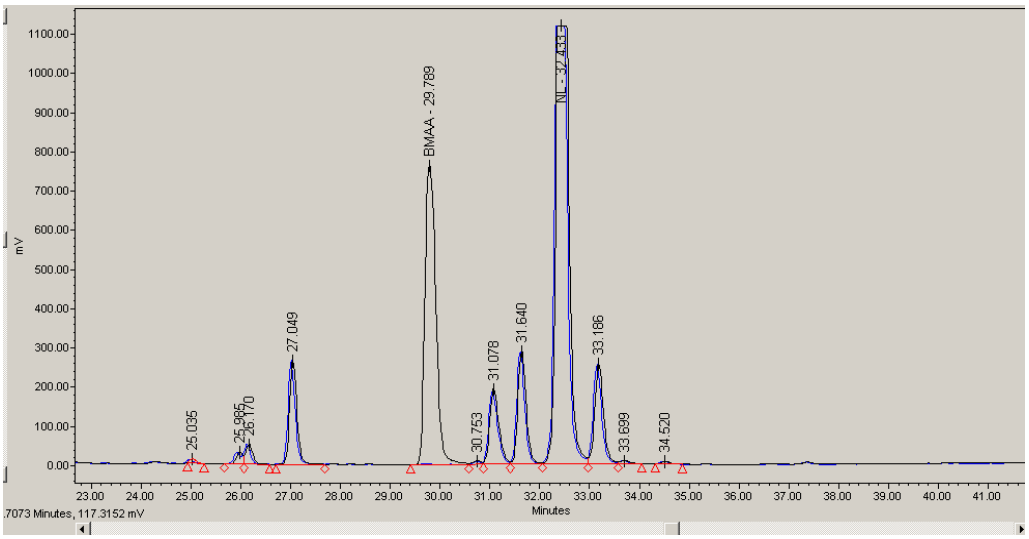



**Figure 21.** Overlayed HPLC-FL profiles of *Planktolyngbya algae* (H) spiked and not spiked with BMAA

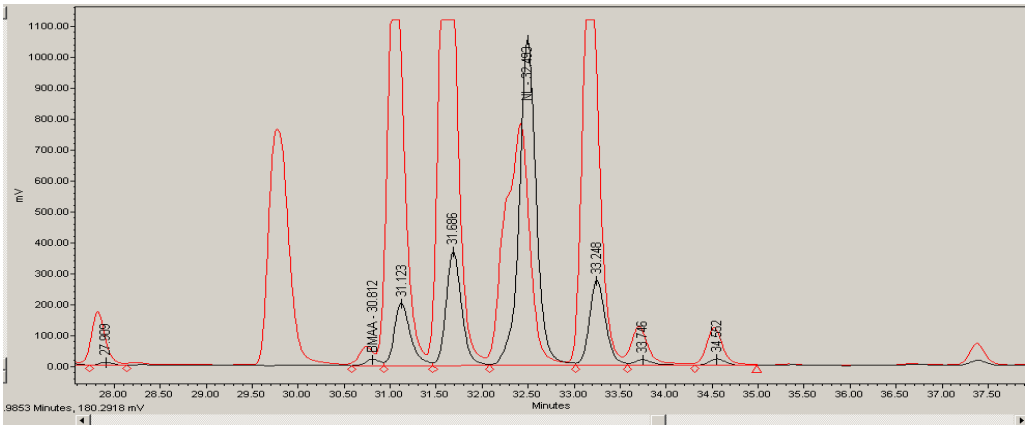



**Figure 22.** Overlayed HPLC-FL profiles of *Phormidium tychomena* (I) spiked and not spiked with BMAA

**Table 8.** Results from the screening of Australian sourced cyanobacteria

| Cyanobacteria | Location in Australia | BMAA concentration |
|---|---|---|
| *Nodularia* (A) | Victoria, 1996 | Not detected (nd) |
| *Microcystis flos aquae* (B) | South Australia, 2002 | nd |
| *Cylindrospermopsis raciborskii* (C) | AWQC Culture #1, 2007 | nd |
| *Cylindrospermopsis raciborskii* (D) | AWQC culture #2, 2007 | nd |
| *Microcystis aeruginosa* (E) | Unknown location | nd |
| *Anabaena circinalis* (F) | South Australia, site #1 | nd |
| *Anabaena circinalis* (G) | South Australia, site #2 | nd |
| *Planktolyngbya algae* (H) | Western Australia | nd |
| *Phormidium tychomena* (I) | Barossa Valley, South Australia | nd |
| *Microcystis wesenbergi* (J) | New South Wales | nd |
| *Benthic oscillatoria* (K) | South Australia | nd |
| *Microcystis flos aquae* (L) | Torrens Lake, South Australia | nd |

# 4 DISCUSSION

The HPLC-FL analytical method of Cox *et al.* (2003) in-conjunction with a modified extraction process developed at the AWQC enabled cyanobacteria to be screened relatively rapidly or the presence of BMAA in levels down to 0.1 ppm (LOD value) or 10 µg/g on a weight per weight basis.

All twelve Australian natural and cultured cyanobacteria samples were negative for the presence of BMAA. These findings differ from three other studies which detected BMAA in samples of cyanobacteria.

Cox *et al.* (2005) reported that freshwater cyanobacteria species originating from six different countries including Australia to be positive for BMAA by HPLC-FL and HPLC-MS analysis (summarised in Table 9).

**Table 9.** Total BMAA levels reported in cyanobacteria species found in international freshwater sources (Cox *et al*. 2005)

| Cyanobacteria species/strain | Origin | Total BMAA (µg/g) |
|---|---|---|
| *Microcystis* PCC 7806 | The Netherlands | 10 |
| *Microcystis* PCC 7820 | Scotland | 18 |
| *Planktothrix agardhii* NIES 595 | Northern Island | 348 |
| *Anabaena variabilis* ATCC29413 | USA | 35 |
| *Anabaena* PCC 7120 | USA | 32 |
| *Cylindrospermopsis raciborskii* CR3 | Australia | 6492 |
| *Nostoc* PCC6318 | Israel | 66 |
| *Nostoc* PCC7107 | USA | 1779 |

BMAA was also detected in cyanobacteria from South Africa by Esterhuizen and Downing (2008) using gas chromatography/mass spectrometry. 96% of the samples from 27 samples which included 11 genera contained BMAA.

Metcalf *et al*. (2008) detected BMAA in 12 samples of cyanobacteria from England, Scotland and Wales using HPLC and mass spectrometry.

There is no clear explanation for the differences in these reports. Regional differences have been recorded in other metabolites produced by cyanobacteria and could also apply to BMAA. Cox *et al.* (2005) did detect BMAA in a culture of *Cylindrospermopsis raciborskii* isolated from a subtropical region in Australia. The *C. raciborskii* culture tested in this study, isolated from temperate South Australia, tested negative for BMAA.

It is also possible that the results may be influenced by differences in the extraction and analytical methods adopted in the different studies.

While the results of this study suggest that BMAA may not be a significant issue for drinking waters in Australia further studies are required.

# 5 SUMMARY AND CONCLUSIONS

A HPLC-FL method for the analysis of BMAA, based on a literature method (Cox *et al*. 2005) and incorporating a modified extraction method, was successfully established at the AWQC. The approximate LOD and LOQ values for BMAA by the AWQC HPLC-FL method were comparable to the literature values (Cox *et al*. 2005). Ten natural and two cultured freshwater cyanobacteria samples originating from Southern Australia tested negative for the presence of BMAA by HPLC-FL and HPLC-MS and therefore did not support the findings of Cox *et al.* (2005) who detected significant concentrations of BMAA in related or analogous cyanobacteria species such as *Cylindrospermopsis raciborskii.*

In conclusion, results of this project demonstrate that BMAA is more than likely not an issue for the Australian water industry at the present time. However, monitoring of cyanobacteria species in particular *Cylindrospermopsis raciborskii* occurring in freshwater sources for the presence of BMAA by HPLC-FL and HPLC-MS/MS should take place in the future to assure that BMAA is not produced as a result of environmental changes (e.g. climate change). In regards to the evaluation of water treatment processes for the removal of BMAA, current data does not justify such a study however it is envisaged that current methods utilising activated carbon for the removal of cyanotoxins (e.g. microcystins) may not be as effective for the adsorption of BMAA from water due to the highly polar nature of BMAA.

# 6 RECOMMENDATIONS

It is proposed that a HPLC-MS method utilising a triple quadrupole MS in the tandem MS mode and/or a time-of-flight MS detector for accurate mass measurement be evaluated for their accuracy to detect and quantify BMAA in environmental samples (e.g. water, cyanobacteria). It is also proposed that cyanobacteria in particular *Cylindrospermopsis raciborskii* that occurs in Australian and international freshwater sources in the future be screened for the presence of BMAA for a three year period by an optimum HPLC-MS method.

# 7 ACKNOWLEDGEMENTS

The project team wishes to thank Geoff Eaglesham and Dr Wasa Wickramasinghe, EnTox, Brisbane, Queensland for the analysis of cyanobacteria extracts by HPLC-MS. Also many thanks to the Cooperative Research Centre for Water Quality and Treatment (CRC WQ&T) and United Water, Australia for providing the funds to carry out this project.

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The research in this document was conducted during the term of the CRC for Water Quality and Treatment and the final report completed under the auspices of WQRA.

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