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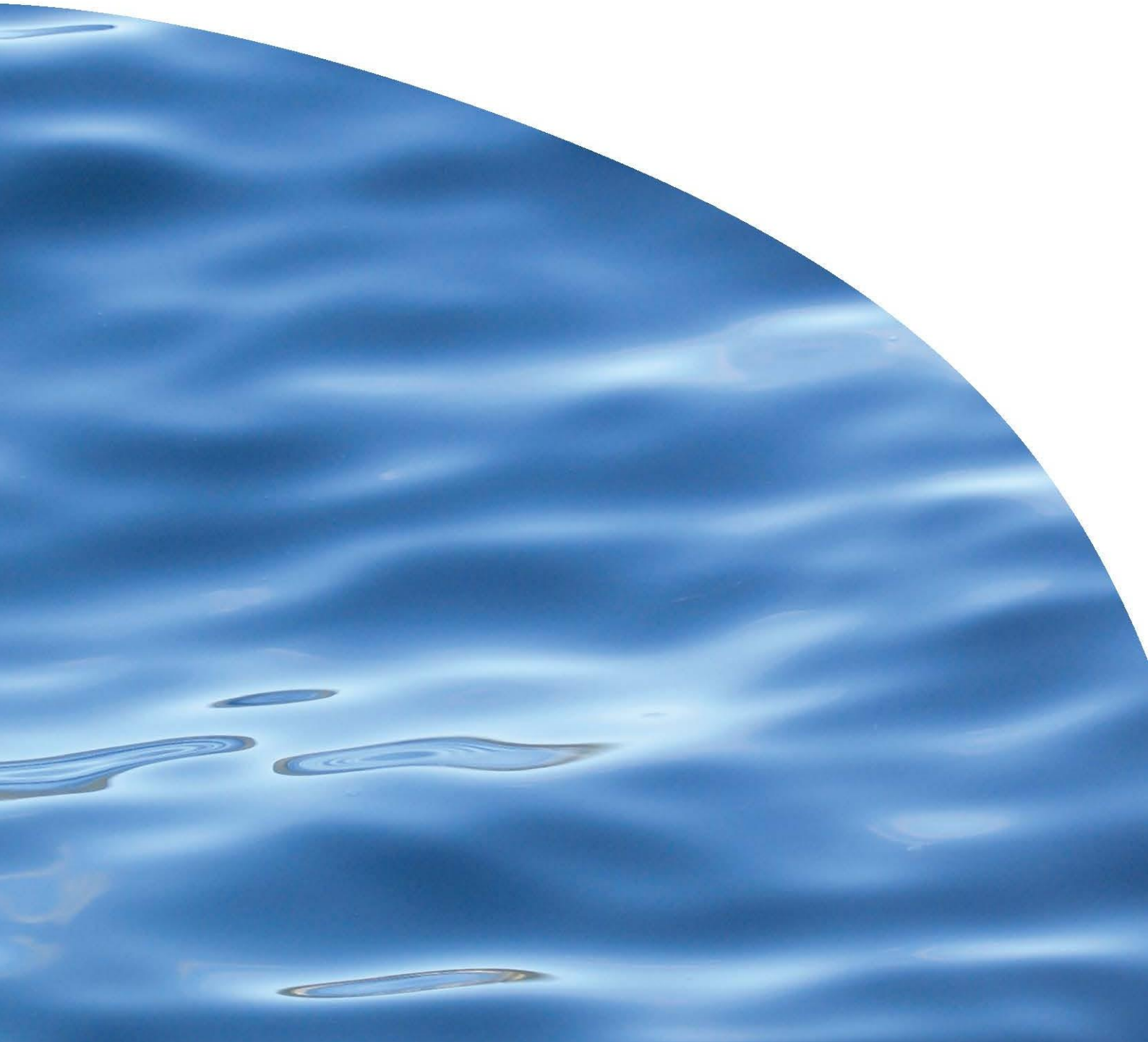
The Acute Toxicity and Biochemical Assessment of Anatoxin-a and Dihydroanatoxin-a



The Acute Toxicity and Biochemical Assessment of Anatoxin-a and Dihydroanatoxin-a

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* Rex Munday sadly passed away on 20 July 2017, but his contribution to this work is gratefully acknowledged

Prepared for the New Zealand Ministry for the Environment

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EXECUTIVE SUMMARY

Phormidium is a cyanobacterium that grows as thick benthic mats and can form blooms in some New Zealand rivers. Over the last decade, the frequency and intensity of blooms has increased. *Phormidium* can produce potent neurotoxins called anatoxins. These blooms pose a health risk to humans and animals, causing multiple dog deaths in the past 15 years. There are inconsistencies and knowledge gaps associated with the toxicological data for the four most prevalent anatoxin congeners in New Zealand rivers; anatoxin-a (ATX), homoanatoxin-a (HTX), dihydroanatoxin-a (dhATX) and dihydrohomoanatoxin-a (dhHTX). This report documents the purification of ATX and dhATX from cyanobacterial material, the assessment of their acute toxicity using mice and their nicotinic acetylcholine receptor (nAChR) binding affinity was determined using a biochemical assay.

Milligram quantities of ATX and dhATX we purified from cyanobacteria. Liquid-liquid partitioning and size-exclusion chromatography was used to purify ATX. Liquid-liquid partitioning and C_{18} chromatography was used to purify dhATX. Both toxins were assessed for purity by nuclear magnetic resonance (NMR) spectroscopy and the amount of toxin was quantified by either liquid chromatography-tandem mass spectrometry (ATX) or quantitative-NMR (dhATX). It was discovered that the dihydro-anatoxin congeners (dhATX and dhHTX) produced by *Phormidium* exist naturally as two structural isomers. An assessment of the isomer composition of the dihydro-anatoxins in *Phormidium* blooms confirmed that the purified dhATX material matched the natural composition. Acute toxicity assessments were conducted in mice by three routes of administration; intraperitoneal (ip) injection, gavage and voluntary consumption. The relative nAChR-binding affinity of the two anatoxin congeners was also assessed using the *Torpedo* electroplaque assay with small modifications to ensure that interferences were avoided.

The median lethal dose (LD_{50}) for ATX in mice by ip injection (0.231 mg/kg; 95% confidence limits of 0.223-0.250 mg/kg) and gavage (10.6 mg/kg; 95% confidence limits of 9.9-12.5 mg/kg) were comparable to data reported previously. A voluntary consumption LD_{50} for ATX was determined for the first time (25 mg/kg; 95% confidence limits of 21.81-34 mg/kg). Whilst the LD_{50} for dhATX by ip injection (0.73 mg/kg; 95% confidence limits of 0.681-0.846 mg/kg) showed it was less toxic than ATX via this route, the LD_{50} values determined by gavage and voluntary consumption (2.5 and 8 mg/kg respectively; 95% confidence limits of 1.99-3.2 mg/kg and 6.34-10.2 mg/kg respectively) demonstrated it was more toxic than ATX by these oral routes. Previously, dhATX was considered to have relatively low toxicity according to ip toxicity work conducted using synthetic material, but the toxicity results from the present study demonstrate that this is not the case when dhATX is orally ingested. The relative nAChR-binding affinity of ATX and dhATX were compared using the *Torpedo* electroplaque assay. Differences observed between the two congeners closely paralleled those observed for the ip toxicity. Despite the ability of this assay to detect these differences, toxicity results conducted *in vivo* (and more specifically by oral administration) are required to provide data for human health risk assessments.

It is anticipated that risk modelling of *Phormidium* blooms will be conducted to evaluate the threat they pose to human health and to assist with the development of meaningful thresholds for *Phormidium* in New Zealand rivers. The acute toxicity values reported here will be required for this modelling. The new information on the oral toxicity of dhATX demonstrates a need for monitoring dhATX in addition to ATX. The viability of conducting toxicity work on dhHTX and HTX should be assessed in the near future.

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GLOSSARY

ATX:	Anatoxin-a
CICCM:	Cawthron Institute Culture Collection of Microalgae
CRM:	Certified reference material
Anatoxins:	Collective term to refer to different anatoxin congeners
DCM:	Dichloromethane
Dihydro-anatoxins	A collective term for dihydroanatoxin-a and dihydrohomoanatoxin-a
dhATX:	Dihydroanatoxin-a
dhHTX:	Dihydrohomoanatoxin-a
DIN:	Dissolved-inorganic nitrogen
DRP:	Dissolved-reactive phosphorous
D_2O:	Deuterium oxide or 'heavy water', a specialised solvent for NMR analysis where the hydrogen atoms have been replaced with deuterium
HTX:	Homoanatoxin-a
IC_{50}:	Concentration where binding is reduced by half
LC-MS/MS:	Liquid chromatography-tandem mass spectrometry
LD_{50}:	Median lethal dose
NRC:	National Research Council
nAChR:	Nicotinic acetylcholine receptor
NMR:	Nuclear magnetic resonance
OECD:	Organisation for Economic Co-operation and Development
prep-HPLC:	Preparative high-performance liquid chromatography
qNMR:	Quantitative nuclear magnetic resonance
Total anatoxins:	The sum of the concentrations of the four anatoxin congeners assessed during this study (ATX, HTX, dhATX and dhHTX)

1. INTRODUCTION

Phormidium is a genus of cyanobacteria that is prevalent in some New Zealand rivers, where it grows as thick benthic mats on the river bed (Figure 1).[1] It is commonly found in cobble-bed rivers, although it is also present in other environments around New Zealand, such as rivers with fine substrate,[2] on the bottom of lakes,[3] and in shallow ponds.[4] Rivers with slightly-elevated dissolved-inorganic nitrogen (> 0.1 mg/L DIN), low dissolved-reactive phosphorous in the water column (< 0.01 mg/L DRP) and higher levels of deposition of fine sediment appear to be more susceptible to *Phormidium* blooms.[1]

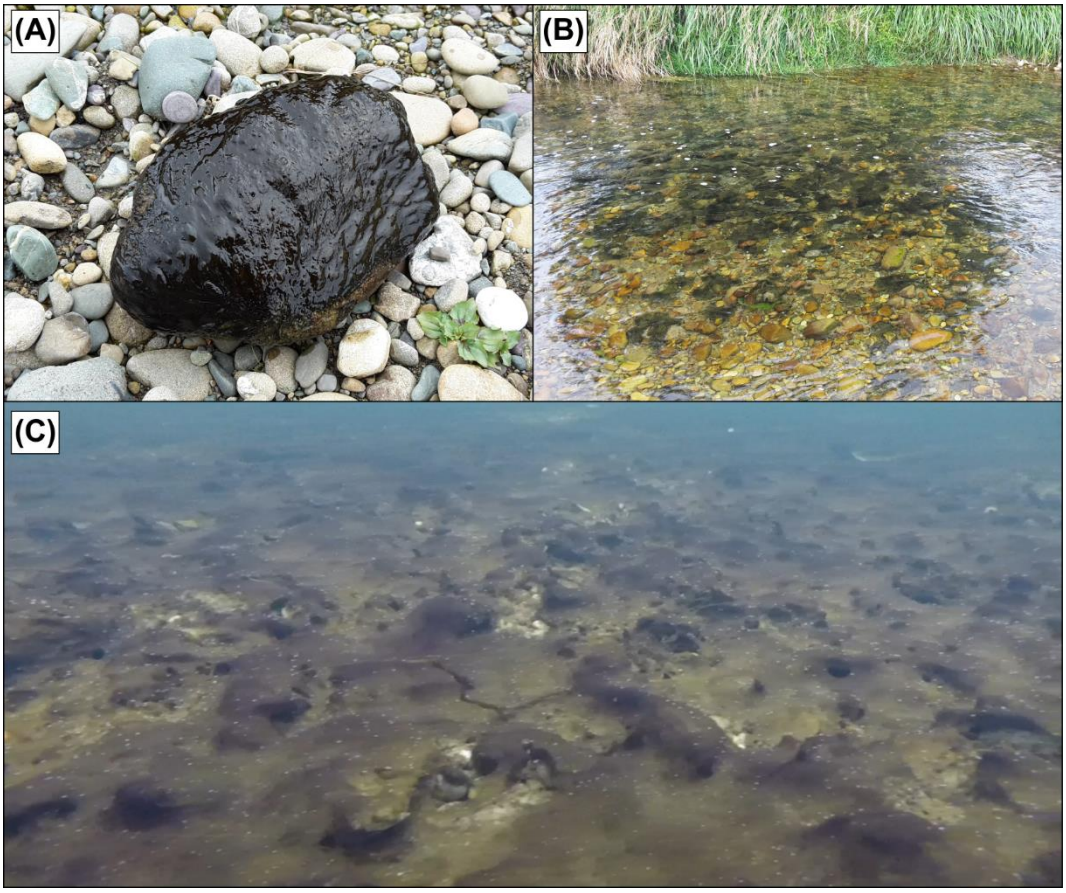

Figure 1: Images of *Phormidium* blooms in New Zealand; **A)** a rock covered in a *Phormidium* mat, **B)** a bloom of *Phormidium* in the Wai-iti River (Tasman region) and **C)** an under-water photo of *Phormidium* mats covering the majority of the surface of the Mataura River (Southland).

Over the last 15 years, *Phormidium* blooms have been associated with multiple dog deaths around New Zealand.[4-6] This is due to the ability of some strains of *Phormidium* to produce potent neurotoxins collectively called anatoxins (Figure 2). The mode of action of these toxins is to block nicotinic acetylcholine receptors (nAChRs) of the post-synaptic membrane at the neuromuscular junction, causing muscular paralysis, leading to death by asphyxia.[7-10]

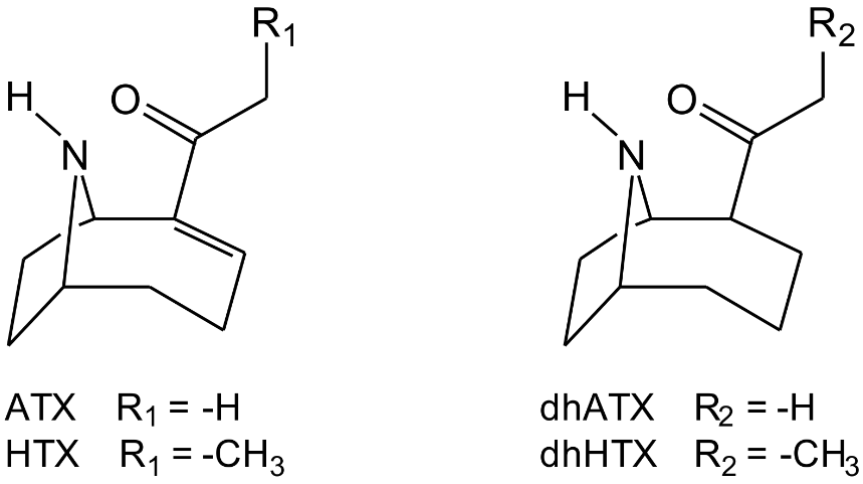

Figure 2: Chemical structures of anatoxin-a (ATX), homoanatoxin-a (HTX), dihydro-anatoxin-a (dhATX) and dihydro-homoanatoxin-a (dhHTX).

The acute toxicity of anatoxin-a (ATX) has been assessed by intraperitoneal (ip) injection and gavage, and on HTX using ip injection (Table 1). The reported ip LD_{50} values range from 0.2-0.375 mg of toxin/kg, and the single ip toxicity measurement for HTX (0.25 mg/kg) was consistent with the measurements for ATX. Other work on the repeated exposure to ATX both orally and by ip/subcutaneous injection are summarised in Cawthron Report No. 2752.[11]

Table 1: Acute toxicity studies performed on anatoxin-a (ATX) and homoanatoxin-a (HTX) in mice.

Compound and 'Form'	Method of Administration	Comments	LD_{50}[a] (mg of ATX / kg)	Ref.
(+)-ATX hydrochloride	ip injection	Commercial ATX product (98% pure).	0.213 (0.197-0.229)	12
'ATX'	ip injection	Commercial ATX product (no information on form or purity).	0.375 (not stated)	13
(+)-ATX hydrochloride	ip injection	Synthetic (+)-ATX (95% pure).	0.316 (0.299-0.334)	14
'ATX'	ip injection	No information on the source, form or purity.	0.2 (not stated)	15
HTX	ip injection	HTX purified from *Oscillatoria formosa* (95% pure).	0.25 (not stated)	16
(+)-ATX hydrochloride	Gavage	Commercial ATX product (98% pure).	13.3 (12.6-13.9)	12

[a] Median lethal dose (LD_{50}) was adjusted for purity and form (when necessary) and 95% confidence intervals are presented in brackets (when reported).

Whilst there is some toxicological data reported for ATX, inconsistencies between the experimental methodologies make reliable interpretation and comparison of the results

difficult. There is much less data for the other anatoxin congeners prevalent in the *Phormidium* blooms that occur in New Zealand; homoanatoxin-a (HTX), dihydroanatoxin-a (dhATX) and dihydrohomoanatoxin-a (dhHTX; Figure 2). The only toxicology information available for the dihydro-anatoxin congeners was generated using synthetic material that does not necessarily match the isomer composition found in the natural environment[15,17] and using an *in vitro* nAChR-binding assay (rather than an *in vivo* assay).[18]

To fill some of these knowledge gaps, the New Zealand Ministry for the Environment (MfE) commissioned a study into the acute toxicity of the anatoxins observed in New Zealand *Phormidium* blooms. To ensure consistency, and relevance to human exposure, toxicity assessments were performed according to the guidelines of the Organisation for Economic Cooperation and Development (OECD; Guideline 425, acute oral toxicity)[19] and using three routes of administration; ip injection, gavage and voluntary feeding. The Abraxis *Torpedo* electroplaque assay was used to measure nAChR-binding affinity. This report documents the acute toxicity work conducted on ATX and dhATX purified from bulk-cultured and wild-harvested cyanobacteria, and how this compares with their nAChR-binding affinity.

2. METHODOLOGY

2.1. LC-MS/MS Analysis of Anatoxins

The concentration of anatoxins in bloom samples, algal cultures, extracts and purification fractions were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Analyses were performed on a Waters Xevo TQ-S mass spectrometer coupled to a Waters Acquity I-Class liquid chromatography system. Sample components (5 µL) were separated using a Thermo Hypersil Gold-aq column (1.9-µm; 50×2.1 mm) at 40°C with a gradient of water + 0.1% formic acid to acetonitrile + 0.1% formic acid at a flow of 0.6 mL/min. Anatoxins were quantified in positive ion mode using multiple reaction monitoring channels for ATX, HTX, dhATX and dhHTX.[4] A certified reference material (CRM) for ATX (National Research Council, Canada) was used to prepare a five-point external calibration curve in 0.1% formic acid (2-100 nM). Unless specifically stated, the concentrations of HTX, dhATX and dhHATX were determined using the ATX CRM calibration curve and a relative response factor of 1. When anatoxin concentrations were outside of the calibration range, samples were diluted with 0.1% formic acid and re-analysed.

2.2. Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was used to assess the purity of fractions resulting from purification procedures, to identify contaminants and confirm the molecular structure of purified compounds. Samples were prepared by freeze-drying an aliquot and resuspending in deuterium oxide or 'heavy water' (D_2O; 0.6 mL, 99.9% deuterium atoms,

from an ampule). The sample solution was placed in a NMR tube and sent to GlycoSyn for analysis (Callaghan Innovation, Lower Hutt).

2.3. Purification of Anatoxin-a from Bulk Cultured Cyanobacteria

In order to produce sufficient ATX to conduct the acute toxicity studies, the cyanobacterium *Cuspidothrix issatschenkoi* (CAWBG-02)[20] was bulk-cultured using a pH-controlled air-lift bag system (Figure 3). Following two weeks of growth, cultures were harvested by acidification with glacial acetic acid (to pH 4.5-5) and addition of a hydrated bentonite solution (0.25% w/v; final concentration). After bubbling for 15 min, the air supply was turned off and the culture was left to flocculate and settle. The concentrated cells were siphoned into 1 L plastic bottles and frozen immediately. Any remaining ATX precursor present in the CAWBG-02 culture material (11-carboxyl-ATX) was converted into ATX by defrosting the frozen cells and incubating at 35°C for 5 h.[20] The cell suspension was concentrated by freeze-drying (Figure 4A) before the dried cells were extracted four times with 0.1% acetic acid (v/v). The clarified cell extract was concentrated to dryness by freeze-drying and stored at −20°C until required. This extract was re-suspended in 0.1% formic acid (v/v) and adjusted to pH ≤ 3.5 with concentrated formic acid. After filtration, the solution was extracted twice with dichloromethane (DCM) in a separating funnel to remove neutral and acidic organic contaminants. The aqueous solution was retained, salt (NaCl) was added and the solution was adjusted to pH 10.5 using a saturated sodium carbonate (Na_2CO_3) solution. The alkaline solution was returned to the separating funnel and extracted four times with DCM to extract the ATX. The DCM extracts were combined and dried over coarse anhydrous sodium sulphate (Na_2SO_4) before the DCM was removed by rotary evaporation.

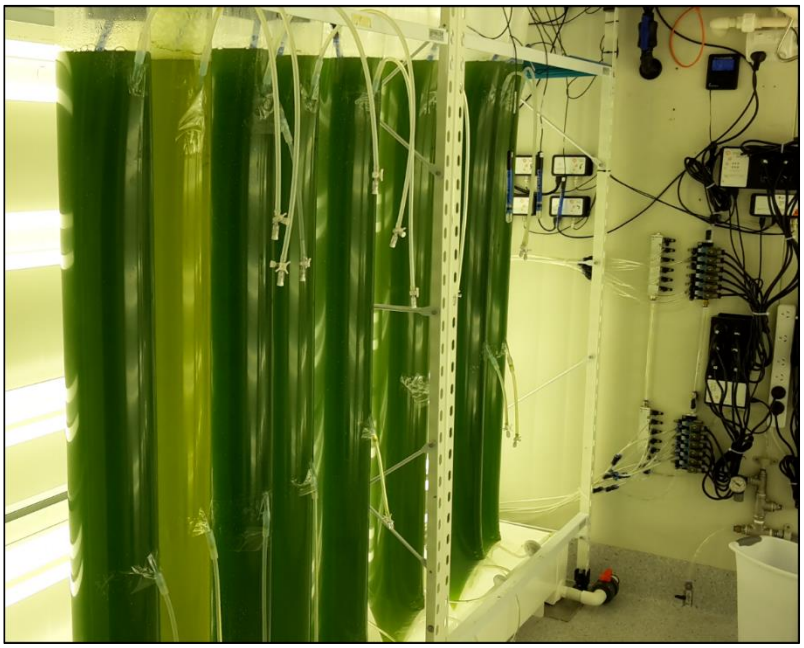

Figure 3: Bulk-culturing of the cyanobacterium *Cuspidothrix issatschenkoi* (CAWBG02) using Cawthron's pH-controlled air-lift bag system in order to produce sufficient anatoxin-a for acute toxicity assessments.

To remove lipophilic impurities, the dried DCM extract was re-dissolved in 0.1% acetic acid (v/v), loaded onto a Strata-X solid-phase extraction cartridge (1 g). The cartridge was washed with 0.1% acetic acid (v/v) followed by three successive washes of 10% methanol + 0.1% acetic acid (v/v). The ATX eluted in the flow-through and the 0.1% acetic acid wash, allowing separation from lipophilic contaminants, which were eluted in the later flushes. The resulting ATX sample was freeze-dried, dissolved in 0.1 M acetic acid and loaded on a 50×500 mm size-exclusion column (Bio-rad P2 gel; Figure 4B). Mobile phase (0.02 M acetic acid at 5 mL/min) was run through the column to elute the loaded compounds. Fractions were assessed by LC-MS/MS to determine where the ATX eluted and the ATX-containing fractions were pooled, supplemented with 0.1% acetic acid (v/v; final concentration) and progressively freeze-dried into smaller vessels (3-times; Figure 4C-E). This resulted in 15.2 mg of yellow oil that was immediately resuspended in 0.1% acetic acid (v/v; 1 mL). Analysis by LC-MS and NMR spectroscopy demonstrated that the purified sample was suitable for toxicity studies. Most of the purified ATX was shipped to AgResearch Ruakura (Hamilton, New Zealand) for acute toxicity assessment and a small aliquot (50 µL) was shipped to Massey University (Wellington, New Zealand) for nAChR-binding affinity assessment using the *Torpedo* electroplaque assay.

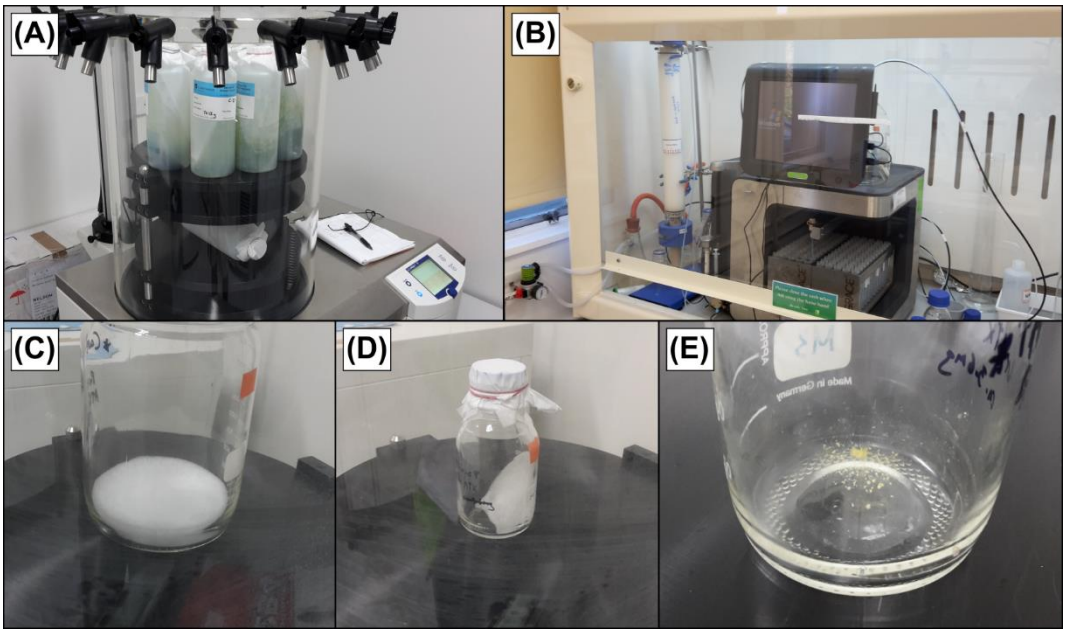

Figure 4: Images from the anatoxin-a (ATX) purification process; **A)** concentration of the harvested cells by freeze-drying, **B)** size-exclusion chromatography to separate ATX from impurities (note the P2 gel column on the left), and **C-E)** sequential freeze-drying of the final product to yield a yellow oil **(E)**.

2.4. Purification of Dihydroanatoxin-a and Dihydrohomoanatoxin-a from *Phormidium* Bloom Material

In order to produce sufficient dhATX and dhHTX (at times referred to collectively as dihydro-anatoxins) to conduct the acute toxicity studies, *Phormidium* bloom material was collected from rivers in the Tasman, Wellington and Canterbury regions.[21] Samples were collected by scraping the *Phormidium* mats from rocks into a net. The *Phormidium* was transferred into buckets, supplemented with glacial acetic acid to a final concentration of 0.1% (v/v) and stored at −20 °C.

Phormidium bloom material from the Wai-iti (ALG-01), Hutt (ALG-10) and Cardrona (ALG-13) Rivers (see Cawthron Report No. 3032 for more details on the samples)[21] was used for the purification of dhATX and dhHTX. This material also contained lower levels of HTX (2%; w/w) and very low levels of ATX (0.03%; w/w). Frozen *Phormidium* material was defrosted at 4°C after being supplemented with 0.1% acetic acid (v/v; final concentration). Once defrosted, the sample was divided into freeze-drying trays, re-frozen at −20°C, and then chilled to −70°C. The samples were freeze-dried over the course of five days before being broken into a coarse powder (1-15 mm pieces), sieved to remove stones and milled in a kitchen blender.

In batches, the milled powder (ca. 2.8 kg total) was extracted in 0.1% acetic acid (v/v) at a 1:10 ratio (*Phormidium* powder / extraction solution). The cells were disrupted by homogenising in the extraction solution using a kitchen blender (high speed for 30 sec) and an Ultra-turrax disperser (17,500 rpm for 2 min). The extract was left for 2 h to allow the dense foam that formed to settle (Figure 5A). The foam was removed using a plastic spatula and the liquid was strained overnight through butter muslin cloth (Figure 5B). To precipitate proteins, the filtered extract was supplemented with chloroform ($CHCl_3$) at a 3:1 ratio (extract / chloroform), shaken and left for 15 min to form a phase partition (Figure 5C). The upper (aqueous) layer was decanted off and retained. The $CHCl_3$ was washed with 0.1% acetic acid (v/v; using 50% of the volume of the original extract). After shaking and allowing to settle, the two aqueous extracts were combined and vacuum-filtered (Whatman GF/F; Figure 5D). The clarified extract was aliquoted into plastic bottles (ca. 500 mL each; Figure 5E) and concentrated twice by freeze-drying (Figure 5F). Following the first freeze-drying step, ten bottles of dried extract were resuspended in 0.1% acetic acid (v/v; ca. 1.1 L), vacuum filtered (GF/F), aliquoted into two bottles and freeze-dried. During this step, the purple pigment abundant in the ALG-10 sample (phycoerythrin) did not re dissolve and was filtered out of the concentrated extract. Seven batches of concentrated anatoxin extract (two bottles described above) were produced from the 2.8 kg of *Phormidium* material extracted.

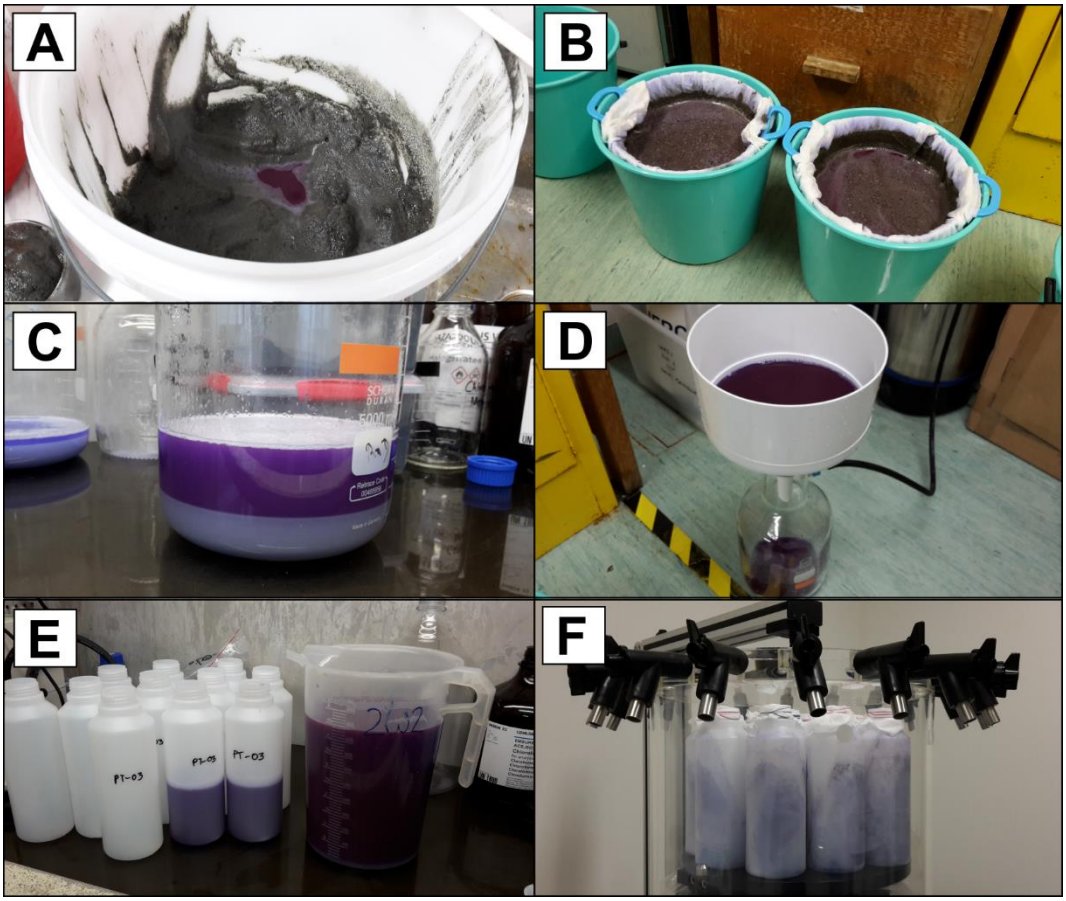

Figure 5: Extraction and concentration of *Phormidium* bloom material for the purification of dihydroanatoxin-a and dihydrohomoanatoxin-a; during the extraction process a thick foam forms which settles on the surface **(A)**, the foam is removed and the liquid is filtered through fine muslin overnight **(B)**, before being shaken with chloroform and allowed to settle into two layers **(C)**, the top layer is filtered through glass-fibre filters **(D)**, dispensed into bottles **(E)** and dried on a freeze-drier **(F)**.

Each batch of concentrated extract (two bottles) was resuspended in 0.1% acetic acid (v/v; 200 mL total) by sonicating in a bath sonicator for 30 min. To precipitate proteins, chilled ethanol (800 mL) was added to the sample and it was left at −20°C overnight. The solution was centrifuged (3,200×g; 10 min; 4°C) and the supernatant was decanted into a round bottom flask. The clarified supernatant was rotary evaporated down to 100 mL to remove the ethanol and stored at 4°C until required. The protein-precipitated anatoxin extract was adjusted to pH ≤ 3.5 using concentrated formic acid and vacuum filtered (GF/F). The filtered solution was extracted three times with DCM in a separating funnel to remove neutral and acidic organics. Severe emulsions generally formed and needed to be broken by adding ethanol. The aqueous solution was retained, NaCl was added (1% w/v, final concentration) and the solution was adjusted to pH 10.5 using a saturated Na_2CO_3 solution. The alkaline solution was vacuum filtered (GF/F), transferred back into the separating funnel and extracted three times with DCM to extract the anatoxins. Severe emulsions generally formed and were broken by adding ethanol. The three DCM-extracts were dried over coarse

anhydrous Na_2SO_4, combined and the DCM was evaporated using a rotary evaporator. The anatoxin material was resuspended in 0.1% acetic acid (v/v; 5 mL).
The ethanol used to break the emulsions reduced the efficiency of the liquid-liquid partitioning, allowing contaminating compounds to migrate into the alkaline DCM phase with the anatoxins. Therefore, the anatoxin fractions where ethanol was used to break emulsions were combined and submitted to the liquid-liquid partitioning clean-up procedure for a second time. Since many of the cellular components had been removed through the initial phase partitioning, severe emulsions did not occur during the second liquid-liquid partitioning.

The resulting extracts from liquid-liquid partitioning were fractionated on a C_{18} Sep-Pak column (25×180 mm; Superflash C_{18}; Agilent Technologies) in three batches (ca. 20 mL each). The extract containing anatoxins was loaded onto the column in 0.1% acetic acid (v/v) and separated isocratically at a flow rate of 20 mL/min for 30 min before progressing to a mobile phase of 20% methanol + 0.1% acetic acid (v/v) in a linear gradient over 30 min. Fractions (10 mL each) were collected in clean 15 mL plastic tubes and assessed by LC-MS/MS to determine where the various anatoxin congeners eluted (dhATX at 4.5 min, dhHTX at 8.5 min and HTX at 9.5-22.5 min). The fractions containing the different anatoxin congeners were pooled, supplemented to 0.1% acetic acid (v/v; final concentration) and progressively freeze-dried into smaller vessels (2-times). The fractions containing dhHTX and HTX were reserved for additional purification.

Analysis of the purified dhATX sample by NMR spectroscopy identified that two additional compounds had been co-purified with the dhATX; phenylethylamine and tyramine (Figure 6). To remove these impurities, the dhATX sample was further fractionated using the C_{18} chromatography system described above, but the fractions were analysed using a LC-MS selected ion recording method to detect the two impurities (phenylethylamine at *m/z* 122.1 and tyramine at *m/z* 138.1). Two fractions that contained the majority of the dhATX, but minimal tyramine and no phenylethylamine, were combined and freeze-dried yielding a yellow oil. Because ATX losses were noted during freeze-drying, the dihydro-anatoxins were never taken to complete dryness during concentration steps.

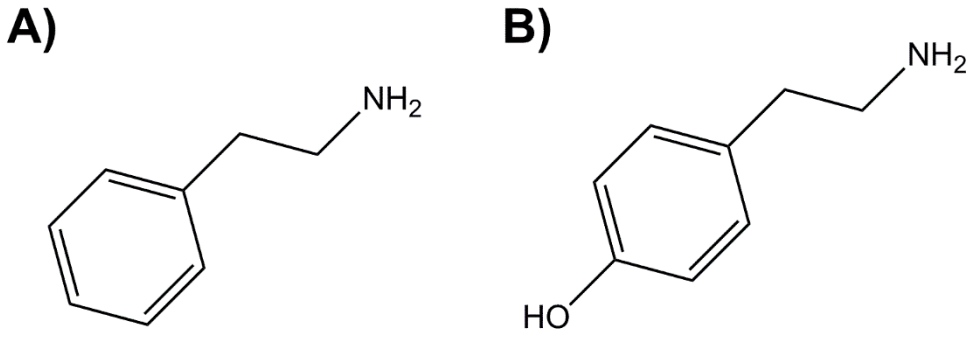

Figure 6: The structures of **A)** phenylethylamine and **B)** tyramine, two impurities removed from the dihydroanatoxin-a material.

The material, comprised of a mixture of dhHTX and HTX, was fractionated again by C_{18} Sep-Pak chromatography (as described above) to remove the HTX, and the fractions that contained only dhHTX were combined with those from the previous purification steps. Analysis of the purified dhHTX sample by NMR spectroscopy indicated that several impurities were still present. Using a similar strategy to that successfully employed during the dhATX purification, the sample was fractionated by C_{18} Sep-Pak chromatography and the fractions were analysed by LC-MS in scan mode (positive ion mode; *m/z* 50-1,000). The spectra for individual fractions were then assessed for the presence of ions not due to dhHTX or background signals. Two candidate ions were identified at *m/z* 249 and 330 and when the fractions were recombined, those that contained the highest levels of these ions were avoided. Whilst this strategy removed some of the contaminating compounds, most still remained.

To remove these persistent impurities, preparative high-performance liquid chromatography (prep-HPLC) was performed using a Synergi C_{18} column (140×21.2 mm, 5-µm; Phenomenex) coupled to a Shimadzu LC-20AP pump, CMB-20A communications module and a SPD-M20A photo-diode array (PDA) detector equipped with a 0.5 mm cell. The dhHTX eluted between 3.5-8.2 min and did not absorb strongly at 210 nm. The PDA trace also contained four distinct peaks at 8.7, 9.4, 11.3 and 13.9 min. The dhHTX fraction and the other peaks were analysed by proton NMR spectroscopy which indicated that whilst the prep-HPLC removed some minor contaminants, the major impurity/ies remained. Further purification of the dhHTX would entail taking additional risks which could result in large losses of the dhHTX partially purified material (5-10 mg, depending on the calibration standard used). Recommendations and risk mitigation strategies to proceed with the purification of dhHTX are detailed in Cawthron Report No. 3182.[22]

2.5. Analytical Assessment of Dihydro-Anatoxin Isomer Composition

2.5.1. NBD-F Derivatisation Method

Samples containing anatoxins were derivatised with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) before analysis by liquid chromatography with fluorescence detection (LC-FLD).[17] Anatoxin extracts were cleaned-up/concentrated by extracting dried *Phormidium* bloom material in 0.1% formic acid (FA) using three freeze-thaw cycles with sonication between, liquid/liquid partitioning of the clarified extract (2 mL) with DCM (5 mL) to remove neutral and acidic organics, adjusting 1 mL of the aqueous component to pH > 10.5 with Na_2CO_3 (0.25 M), liquid/liquid partitioning with DCM (5 mL) to collect the anatoxins in the DCM fraction and drying under a stream of nitrogen gas at 40°C. The cleaned-up sample was resuspended in a tetraborate solution (0.1 M; 500 µL), and an aliquot (100 µL) was mixed with NBD-F derivatisation reagent (1 mg/mL in acetonitrile; 50 µL) and incubated at ambient temperature for 10 min before the reaction was stopped using hydrochloric acid (HCl; 1 M; 50 µL). The derivatisation products were analysed by LC-FLD using a Develosil RP-Aqueous C_{30} column (150 × 1 mm, 3-µm; Phenomenex) and a gradient of 35-50%

acetonitrile over 14 min. Fluorescence measurement used excitation at 470 nm and monitored light emission at 530 nm.

2.5.2. LC-MS Isomers Method

A simplified extraction and analysis procedure, where *Phormidium* material was extracted in water and analysed directly by LC-MS, was developed in order to overcome some of the problems experienced with the NBD-F derivatisation method. Freeze-dried *Phormidium* samples (ca. 50 mg) were extracted in milli-Q water (1 mL) using three freeze-thaw cycles with sonication (30 min) between each. The clarified extract (2 µL injection) was analysed by LC-MS using a Hypersil Gold aQ column (Thermo Scientific; 50 × 210 mm, 1.9-µm) and a gradient of 0-40% acetonitrile (mobile phases contained 0.01% formic acid; v/v) over 2 min. Mass spectrometry detection used positive mode electrospray ionisation and the following multiple-reaction monitoring transitions; 168.1 > 56.0 (dhATX), 182.1 > 57.0 (dhHTX) and 182.1 > 98.0 (dhHTX).

2.6. Toxicological Evaluation

The acute toxicities of the purified ATX and dhATX were determined according to the principles of OECD Guideline 425,[19] and the LD_{50} value for each was calculated using the computer programme associated with this guideline.[23] All animal experiments were approved by the Ruakura Animal Ethics Committee. Anatoxin solutions were diluted with 1 mM HCl for dosing. The volume administered by ip injection was 1 mL and by gavage was 0.2 mL. Tap water and food (rat and mouse cubes; Speciality Feeds Ltd., Australia) were available to the mice both before and after dosing. The mice were monitored intensively during the day of dosing, and survivors were subsequently examined daily. Body weights were recorded each day. At 14 days after dosing, survivors were euthanised using carbon dioxide inhalation and necropsied. The weights of the liver, lungs, spleen, kidneys, heart, stomach and whole gut of all the mice were recorded at necropsy, and relative organ weights were calculated as a percentage of body weight.

To conduct the voluntary feeding experiments mice were trained to eat cream cheese. After a period of training, mice happily and rapidly consumed small quantities of cream cheese. However, once ATX was added they refused to eat this mixture. This appeared to be due to ATX itself rather than due to the dissolution matrix (dilute acetic acid), so it is possible that the ATX had an unfamiliar taste or smell that deterred the mice. To overcome this, the ATX was mixed with a small amount of ground mouse food in a glass positive displacement pipette tip. The mixture was placed onto the animal's tongue causing them to swallow the dose. This method worked well and was used for all subsequent voluntary feeding experiments.

2.7. *Torpedo* Electroplaque Assay

The Abraxis *Torpedo* electroplaque assay is a commercial application of the method of Rubio et al, 2014[24] that assesses the binding affinity of anatoxins to nAChRs. *Torpedo* electroplaque, which is rich in nAChRs, is used to coat the bottom of the wells in a 96-well plate. Once the test material has been given time to bind to the receptors, another nAChR-binding toxin, α-bungarotoxin (coupled to biotin), is added, which can only bind to unoccupied receptors. After further incubation, all solutions are washed out and the amount of bound α-bungarotoxin-biotin is assessed using horse-radish peroxidase-coupled avidin (which binds tightly to biotin). It is the amount of horse-radish peroxidase that can be measured, by addition of a substrate, and measuring the amount of coloured product that results. With increasing anatoxin-binding, the amount of colour reagent produced decreases as less α-bungarotoxin could bind. The colour produced is measured at a wavelength of 450 nm, after acidification of each well to stop the reaction from continuing.

Dilution curves for the purified ATX and dhATX were generated. Each was plotted as % binding against the log of the calculated concentration (in ppb). The IC_{50} for each toxin was calculated using a four-parameter fit, using Graphpad Prism software. Additionally, the results were assessed in comparison to the standard curve obtained using the kit standards, to give ATX-equivalent values for the different toxin dilutions.

3. RESULTS

3.1. Purification and Characterisation of Anatoxin Congeners

3.1.1. Anatoxin-a Material

The purified ATX sample was a yellow oil (15.2 mg) that was immediately re-suspended in 0.1% acetic acid (1 mL) and analysed by LC-MS/MS. The LC-MS/MS quantitation indicated that there was 6 mg of ATX in the sample. The overall purity for the ATX-acetate (the salt produced when the ATX is dried in acetic acid; 8.2 mg) was 54% (w/w of the total mass). Analysis also demonstrated that there was a small amount of dhATX in the sample (0.06% of the total anatoxins; ATX + dhATX).[25]

Nuclear magnetic resonance spectroscopy was conducted to; **a)** unequivocally confirm that the purified compound was ATX, **b)** determine the level of residual acetic acid present in the sample, and **c)** identify if other organic impurities were present in the sample. The proton NMR spectrum contained several clearly observable peaks from the ATX structure; namely, the H3 alkene proton at 7.33 ppm, the H1 methine at 4.88 ppm, the H6 methine at 4.14 ppm, and the H10 methyl at 2.22 ppm (Figure 7). The integrated peak area for these four signals were as expected according the number of protons present in the structure; the H3 peak was 1.00 (one proton), the H1 peak was 0.98 (one proton), the H6 peak was 1.08 (one proton) and the H10 peak was 2.9 (three protons). The multiplet signals observed

between 1.7-2.6 ppm were similar to the four methylene chemical environments found in ATX but were not able to be assigned using the current NMR data.

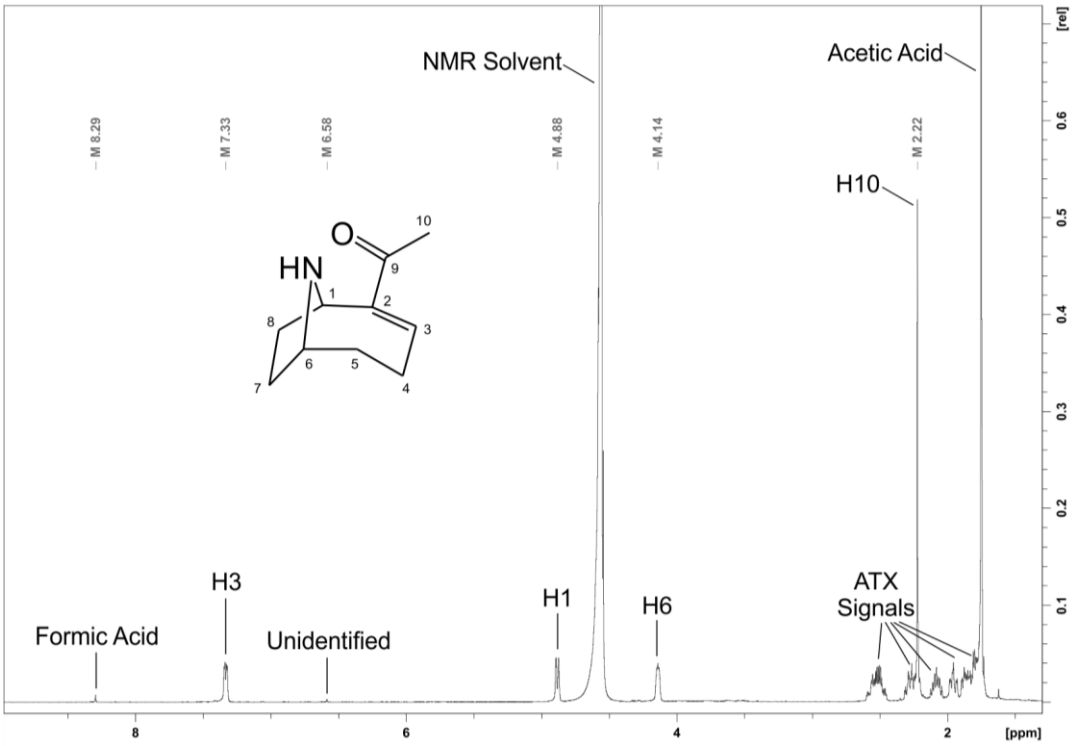

Figure 7: Proton nuclear magnetic resonance (NMR) spectrum for the purified anatoxin-a (ATX) sample dissolved in D_2O and the structure of ATX showing the numbering used for the NMR assignment.

The only other major signal observed in the NMR spectrum for the ATX sample was a singlet at 1.75 ppm, representing the methyl protons of acetic acid (Figure 7). The peak area from this signal compared to that of the ATX H10 methyl signal (2.22 ppm) indicated that the sample contained 5.5 mg of residual acetic acid (using the molar amount of ATX present). Minor signals were observed at 8.29 ppm and 6.58 ppm. The 8.29 ppm signal was likely to be due to the methine proton of formic acid and would equate to 0.3% of the sample mass (based on its peak area). The 6.58 ppm signal was not identified but (based on its peak area) would equate to a very small proportion of the sample mass (<0.5%).

Based on the mass of ATX, and the residual acetic acid and formic acid, 75.4% (w/w) of the sample mass that was measured gravimetrically was accounted for. As ATX losses were observed during freeze-drying, the sample cannot be exhaustively dried and it was likely that residual water is also present. As no major organic impurities (besides acetic acid) were observed in the NMR analysis, acute toxicity work was conducted using dosages calculated from the ATX concentration that was determined by LC-MS/MS (5,953 µg/mL).

3.1.2. Dihydro-anatoxin-a Material

The purified dhATX sample was a yellow oil which was immediately re-suspended in 0.1% acetic acid (1.7 mL). The proton NMR spectrum of the material contained several clearly-observable peaks from the dhATX structure; the H6 methine at 4.20 ppm, the H2 methine at 3.21 ppm, the H1 methine signals at 4.35 and 4.52 ppm (due to two dhATX isomers being present), and the H10 methyl signals at 2.31 and 2.34 ppm (due to two dhATX isomers being present; Figure 8). During the purification, two impurities were identified; phenylethylamine and tyramine. No signals for phenylethylamine were observed in the proton NMR spectrum for the purified dhATX material and the intensity of the tyramine signals was low (2.98, 3.29, 6.96 and 7.27 ppm). Residual acetic acid (1.97 ppm) and formic acid (8.51 ppm; Figure 8) was also observed.

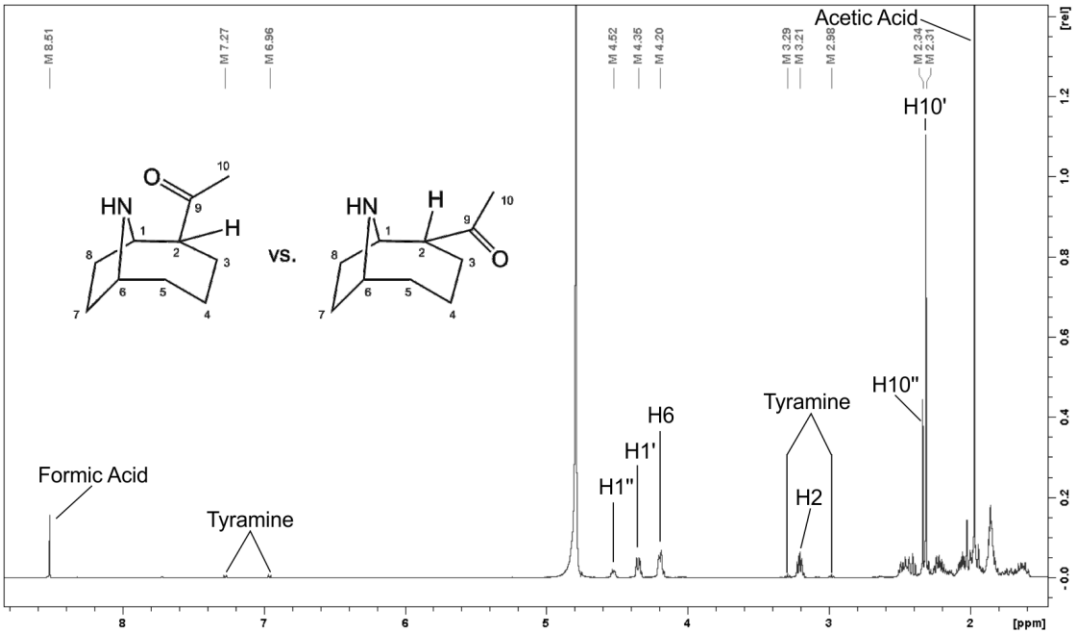

Figure 8: Proton nuclear magnetic resonance (NMR) spectrum for the purified dihydroanatoxin-a (dhATX) dissolved in D_2O and the putative structures of the two dhATX isomers showing the numbering used for the NMR assignment.

When the dhATX sample was left in NMR solvent (D_2O) for a week and re-analysed, several of the dhATX signals had reduced in intensity; H10', H10'' and H2 (2.31, 2.34 and 3.21 ppm respectively). This was due to isomerisation of the compound leading to deuterium atoms being exchanged for protons. Because deuterium nuclei are not observable by NMR spectroscopy, the intensity of these signals was reduced. This also affected the ratio of the H1' and H1'' NMR signals as the isomer composition had changed.

Because reference material for dhATX is not available, the amount of dhATX present in the purified material could not be accurately quantified using LC-MS. Instead, the concentration of dhATX and the impurities present in the sample were quantified by quantitative-NMR (qNMR). Triplicate aliquots of the purified dhATX sample (0.04 mL each) were freeze-dried and resuspended in 0.5 mL of D_2O containing an internal standard (maleic acid at 3.45 mM;

TraceCERT® Lot Number BCBV7235, Sigma-Aldrich). The triplicate samples were analysed by proton NMR spectroscopy and the peak integral of the maleic acid signal (6.37 ppm) was compared to the peak integrals from the compounds of interest (dhATX, acetic acid, formic acid and tyramine). Using the concentration of maleic acid in the sample and knowledge of the number of protons present, a molar concentration in the NMR sample was determined for each compound and converted to a mass concentration (mg/mL), using the molar mass of each compound. The concentration was then adjusted for the dilution factor introduced when preparing the sample for qNMR analysis (0.5 mL ÷ 0.04 mL = 12.5× dilution). Because of the deuterium exchange observed in dhATX (described above), only the H1 and H6 signals were used for the quantitation. Because the 2.98 and 3.29 ppm tyramine signals were affected by the dhATX H2 proton signal, only the 6.96 and 7.27 ppm signals were used for the quantitation. Once undertaken, the concentrations determined using the different dhATX protons were comparable, so an average of the H6 signal and the sum of the two H1 signals was used to determine a dhATX concentration of 14.67 mg/mL in the purified sample (Table 2). Repeatability of the three replicates analysed by qNMR was good (3-6% relative standard deviation, RSD; depending on the signal used). The concentration of acetic acid, formic acid and tyramine present in the sample was 4.32, 0.71 and 0.13 mg/mL respectively.

Table 2: Quantitative nuclear magnetic resonance (NMR) spectroscopy analysis of the purified dihydro-anatoxin-a (dhATX) material, where dhATX and impurities (acetic acid, formic acid, tyramine) were quantified against an internal standard at a known concentration (maleic acid).

NMR Signal	No. of ^{1}Hs	Molar Mass (g/mol)	Peak Integral			Conc in NMR Tube (mM)				Conc. in Sample (mg/mL)	Purity (w/w)
			A	B	C	A	B	C	Ave		
Maleic Acid (6.37 ppm)	2	116.07	1.00	1.00	1.00	3.450	3.450	3.450	3.450	NA	NA
dhATX H6 (4.20 ppm)	1	167.25	1.01	1.01	0.95	6.969	6.969	6.555	6.831		
dhATX H1 Iso 1 (4.35 ppm)	1	167.25	0.70	0.71	0.64	4.830	4.899	4.416	4.715	14.67	74.0%
dhATX H1 Iso 2 (4.52 ppm)	1	167.25	0.37	0.36	0.35	2.553	2.484	2.415	2.484		
Acetic Acid (1.97 ppm)	3	60.05	2.76	2.47	2.27	6.348	5.681	5.221	5.750	4.32	21.8%
Formic Acid (8.51 ppm)	1	46.03	0.19	0.19	0.16	1.311	1.311	1.104	1.242	0.71	3.6%
Tyramine (7.27 ppm)	2	137.18	0.03	0.02	0.02	0.104	0.069	0.069	0.081	0.13	0.6%
Tyramine (6.96 ppm)	2	137.18	0.02	0.02	0.02	0.069	0.069	0.069	0.069		

No. of ^{1}Hs = Number of protons. Ave = Average. Conc. = Concentration.

The NMR analysis demonstrated that the dhATX purity was 74% and since the other components were relatively benign the material was of a suitable purity for toxicology evaluation. As described in Section 3.2, the isomer composition of the purified dhATX was consistent with that in the *Phormidium* from the natural environment. Dosages for the acute

toxicity work in mice and the *in vitro* receptor-binding work were calculated using the dhATX concentration determined by qNMR (14.67 mg/mL).

3.2. Assessment of the Isomer Composition of Dihydro-Anatoxins

During the purification work described in Section 3.1.2, it became evident that the dhATX produced by *Phormidium* existed as a mixture of two isomers. Due to the structure of dhHTX and another study,[17] it was highly likely that the dhHTX produced by *Phormidium* also exists as a mixture of two isomers. For the toxicology work to be conducted on dhATX, this raised new questions which needed to be addressed:

- What is the natural dihydro-anatoxin isomer composition in *Phormidium* blooms?
- Does the purified dihydro-anatoxin material produced reflect the natural situation?

To better understand the natural isomer composition of dihydro-anatoxins in *Phormidium* blooms, two analytical methods with different attributes were set-up and used to analyse *Phormidium* extracts and purified anatoxin material.

3.2.1. NBD-F Derivatisation Method

A method to assess the dihydro-anatoxin isomer composition using a NBD-F derivatisation procedure with LC-FLD was initially set up.[17] For this, anatoxin samples were cleaned-up/concentrated using liquid/liquid partitioning prior to derivatisation and LC-FLD analysis. The two dhATX isomers were arbitrarily assigned according to their retention times, with Isomer 1 (7.25 min) eluting earlier than Isomer 2 (7.71 min; Figure 9). Because a suitably pure reference material was not available for dhHTX, isomers of this compound could not be assessed using this method.

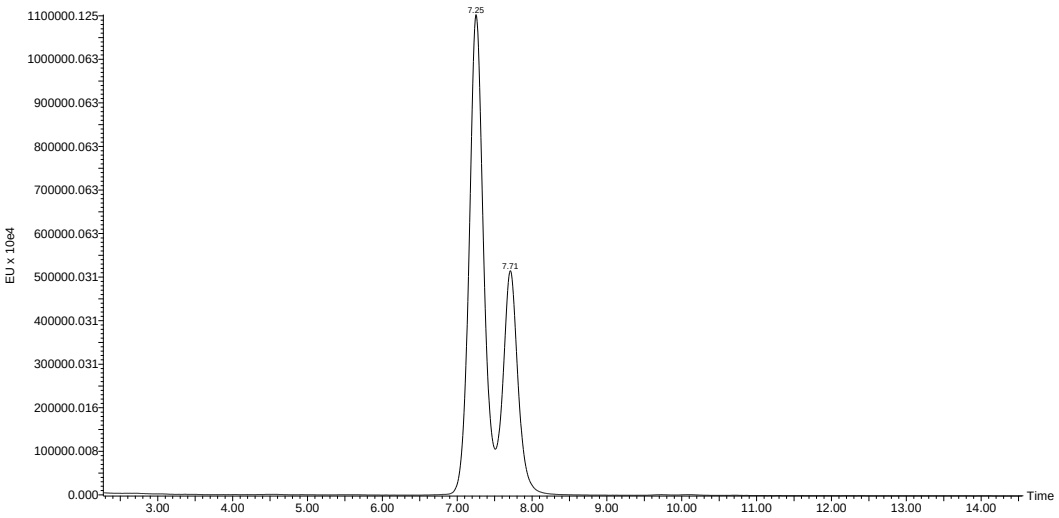

Figure 9: Liquid chromatography-fluorescence detection chromatogram for purified dhATX using the NBD-F derivatisation method (470 nm excitation, 530 nm emission).

To determine the method repeatability, six replicates of a purified dhATX sample and a *Phormidium* extract (ALG-10)[26] were cleaned-up/concentrated, derivatised with NBD-F and analysed by LC-FLD. This indicated that the NBD-F derivatisation method had low repeatability (≥18% RSD; Table 3). Because of this, it was decided that each *Phormidium* bloom extract should be assessed in triplicate to mitigate some of the observed variability. Using the NBD-F derivatization method, the average isomer ratio for the purified dhATX material (2.2; Isomer 1 / Isomer 2; Table 3) was similar to that observed using NMR spectroscopy (1.9; Isomer 1 / Isomer 2).

Table 3: Method repeatability for the dihydro-anatoxin-a (dhATX) isomer composition of purified dhATX and a *Phormidium* extract (ALG-10) using the NBD-F derivatisation method.[a]

Replicate	dhATX Isomer Ratio[b]	
	Purified dhATX	ALG-10 Extract
A	2.5	2.6
B	1.2	4.0
C	2.2	3.8
D	2.1	3.2
E	3.1	4.5
F	2.4	3.9
Ave	2.2	3.7
SD	0.6	0.7
RSD	28%	18%
Min	1.2	2.6
Max	3.1	4.5

[a] An analysis method based on the derivatisation of anatoxins using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) following a sample clean-up/concentration step. [b] Isomer ratio was Isomer 1 / Isomer 2, calculated using peak areas. Ave = Average, SD = Standard deviation, RSD = Relative standard deviation (SD ÷ Ave × 100%), Min = Minimum value, Max = Maximum value.

When *Phormidium* mat samples were extracted and analysed using the NBD-F derivatisation method, all contained Isomer 1 as the predominant dhATX isomer (Table 4). Between the six samples assessed from five different rivers, there was a spread in the average dhATX isomer ratio; 3.7-7.9 (Isomer 1 / Isomer 2). In addition, two thirds of the samples analysed using the NBD-F derivatisation method exhibited high levels of variability (>10% RSD).

Table 4: Analysis of the dihydro-anatoxin-a (dhATX) isomer composition in *Phormidium* bloom samples using the NBD-F derivisation method.[a]

River [b]	dhATX Isomer Ratio [c]		
	Ave	SD	RSD
Hutt River – 2011 (ALG-10)	3.7	0.7	18%
Wai-iti River - December 2012	3.8	0.2	4%
Cardrona River - April 2017	4.3	0.5	12%
Mataura River - April 2017	4.8	0.5	11%
Temuka River - April 2016	5.4	0.8	14%
Hutt River - November 2017	7.9	0.1	2%

[a] An analysis method based on the derivatisation of anatoxins using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) following a sample clean-up/concentration step. [b] Samples are ordered according to the dhATX isomer ratio. [c] The isomer ratio was Isomer 1 / Isomer 2, calculated using peak areas. Ave = Average, SD = Standard deviation, RSD = Relative standard deviation (SD ÷ Ave × 100%).

During the subsequent purification of the dhATX, it was identified that the isomer composition of dhATX was more easily influenced by acid and base than we had initially thought. This was consistent with the work of Méjean *et al* (2016),[27] who showed that pH could influence the isomer composition of dhATX. As the NBD-F derivatisation method uses acidic and alkaline solutions during the clean-up / concentration, and in the derivatisation procedure, this may have influenced the isomer composition and led to some of the observed variability. For this reason, and because the cumbersome sample preparation procedure restricted the number of samples that could be analysed, an alternative method of assessing the dihydro-anatoxin isomer composition was pursued.

3.2.2. LC-MS Isomers Method

In order to overcome some of the hurdles of the NBD-F derivatisation method, a simplified procedure where *Phormidium* material was extracted in water and analysed directly by LC-MS/MS was developed. Whilst there was difficulty separating the two underivatised dhATX isomers, there were advantages in this approach being able to be detect isomers of dhHTX, a reduction in methodology-induced artefacts and, potentially, reduced variability through the simplified sample preparation. As before, the two sets of isomers were assigned according to their retention times; dhATX Isomer 1 (1.43 min), dhATX Isomer 2 (1.64 min), dhHTX Isomer 1 (1.85 min), dhHTX Isomer 2 (1.96 min; Figure 10). Because the compounds were identified by their mass-to-charge ratio and their fragmentation pattern, the dhHTX isomers were able to be assigned in the absence of reference material (unlike with the NBD-F derivatisation method).

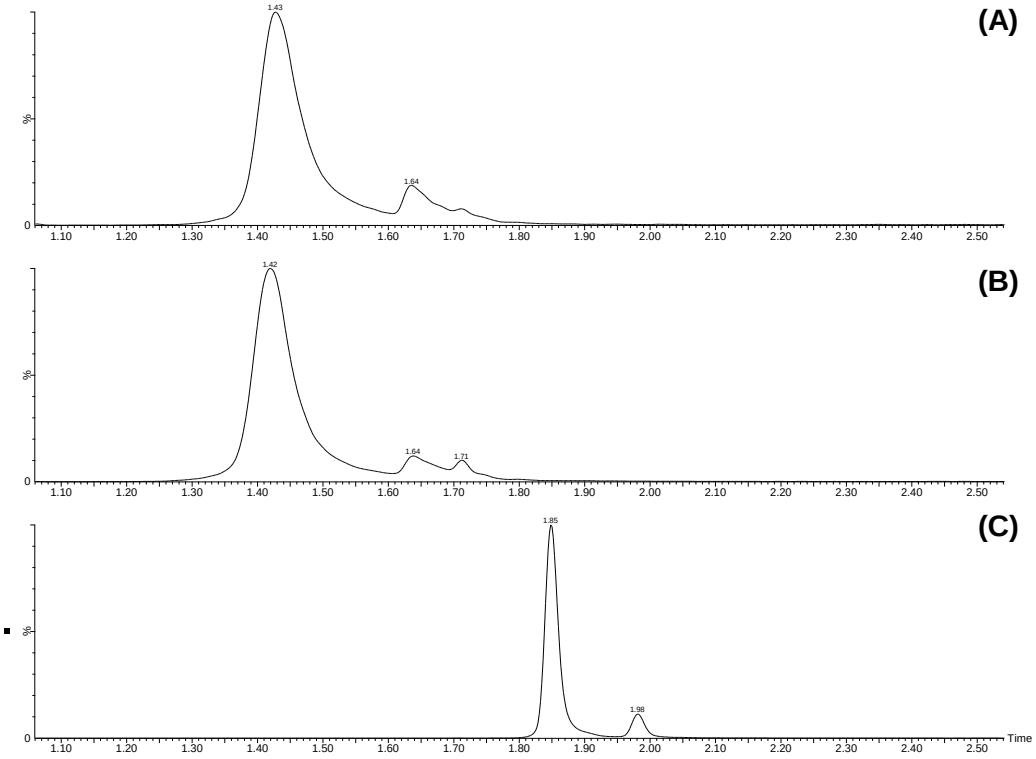

Figure 10: Liquid chromatography-mass spectrometry chromatograms of **A)** the purified dhATX material (only dhATX was present) and a *Phormidium* extract assessing for the presence of **B)** dhATX and **C)** dhHTX.

The method repeatability was assessed using eight individual extracts of the same freeze-dried *Phormidium* material. This yielded less variable results for the isomer ratio of dhATX (5.4% RSD; Table 5) than the NBD-F method, but the dhHTX results still showed high levels of variability (12.4% RSD). It was thought that the poor repeatability for dhHTX was due to the low levels of dhHTX Isomer 2 in this sample, increasing the level of background noise affecting the signal integration. To investigate this further, replicates of the ALG-10 extract that contained higher levels of the dhHTX Isomer 2 were analysed. For this sample, the repeatability of the isomer ratio for dhHTX was more acceptable (4.8% RSD; Table 5).

Table 5: Method repeatability for the dihydro-anatoxin-a and dihydro-homoanatoxin-a (dhATX and dhHTX) isomer composition of a *Phormidium* sample (Hutt River - 2017) and dilutions of the ALG-10 extract using the LC-MS isomers method.[a]

Replicate	Hutt River sample[b]		ALG-10 Extract[b]	
	dhATX	dhHTX	dhATX	dhHTX
A	8.5	13.2	7.6	8.3
B	8.4	11.3	8.2	9.3
C	9.4	11.5	7.7	9.2
D	8.9	12.8	7.8	9.2
E	8.4	16.4	7.7	9.2
F	8.1	14.6	7.8	9.2
G	8.3	13.5	7.7	8.4
H	8.0	13.8	7.3	8.5
Ave	8.5	13.4	7.7	8.9
SD	0.5	1.7	0.3	0.4
RSD	5.4%	12.4%	3.4%	4.8%
Min	8.0	11.3	7.3	8.3
Max	9.4	16.4	8.2	9.3

[a] An analysis method where dihydro-anatoxin isomers are analysed underivatised using liquid chromatography-mass spectrometry (LC-MS). [b] The isomer ratio was Isomer 1 / Isomer 2, calculated using peak areas. Ave = Average, SD = Standard deviation, RSD = Relative standard deviation (SD ÷ Ave × 100%), Min = Minimum value, Max = Maximum value.

Because of the simplified sample preparation, more samples could be analysed using the LC-MS isomers method compared to the NBD-F derivatisation method. To assess the dihydro-anatoxin isomer composition expected in *Phormidium* mats from New Zealand rivers; 37 samples from 12 rivers were analysed for the isomer ratio of the dhATX and dhHTX present.

In the twelve rivers assessed, the dhATX isomer ratio ranged from 2.9-12.2 Isomer 1 / Isomer 2 (44% RSD; Table 6) and the dhHTX isomer ratio ranged from 3.9-13.7 Isomer 1 / Isomer 2 (36% RSD). When multiple samples were analysed from the same river, the average of the replicates was used for the river survey. The large range in dihydro-anatoxin isomer ratios observed was similar to that observed using the NBD-F derivatization method; 3.7-7.9 Isomer 1 / Isomer 2 (32% RSD; Table 4). A large spread in dihydro-anatoxin isomer ratios being determined using two different analytical methods provides strong evidence that the isomer composition of dhATX and dhHTX in *Phormidium* blooms is not fixed.

Table 6: Isomer composition of dihydroanatoxin-a and dihydrohomoanatoxin-a (dhATX and dhHTX) in *Phormidium* samples from twelve New Zealand rivers.[a]

River [b]	Isomer Ratio [c]	
	dhATX	dhHTX
Pareora River (Canterbury)	2.9	nd
Temuka River (Canterbury)	3.5	nd
Makotuku River (Manawatu)	3.5	nd
Mataura River (Southland)	5.7	8.3
Mangatainoka River (Wairarapa)	6.0	13.7
Ashley River (Canterbury)	6.2	8.2
Kaiapoi River (Canterbury)	6.2	3.9
Hutt River (Wellington)	6.5	10.4
Cardrona River (Wanaka)	6.9	10.8
Selwyn River (Canterbury)	8.2	nd
Opihi River (Canterbury)	11.7	nd
Orari River (Canterbury)	12.2	nd
Ave	6.6	9.2
SD	2.9	3.3
RSD	44%	36%
Min	2.9	3.9
Max	12.2	13.7

[a] Analysed using the liquid chromatography-mass spectrometry (LC-MS) isomers method, where dihydro-anatoxin isomers are assessed underivatised. [b] Samples are ordered according to the dhATX isomer ratio. [c] Isomer ratio was Isomer 1 / Isomer 2, calculated using peak areas. nd = not detected, Ave = Average, SD = Standard deviation, RSD = Relative standard deviation (SD ÷ Ave × 100%), Min = Minimum value, Max = Maximum value.

To assess whether the dihydro-anatoxin isomer composition was constant in a river or changed during a bloom season, a sample set collected from the Ashley River (Canterbury) over a 15-week period was assessed using the LC-MS isomers method. In this sample set, there was less variability in isomer ratio than was observed amongst New Zealand Rivers for both dhATX (4.0-9.4 Isomer 1 / Isomer 2; 21% RSD) and dhHTX (5.2-11.4 Isomer 1 / Isomer 2; 22% RSD; Table 7). When the isomer composition data was assessed graphically (Figure 11), there may be an increasing trend in the dhHTX isomer ratio (Isomer 1 / Isomer 2) over the 15-week sampling period and potentially an increasing and then deceasing trend in the dhATX isomer ratio (Isomer 1 / Isomer 2). Trend analysis was not conducted to assess this further.

Table 7: Isomer composition of dihydro-anatoxin-a (dhATX) and dihydro-homoanatoxin-a (dhHTX) in *Phormidium* samples collected over a 15-week period from the Ashley River (Canterbury).[a]

Sampling Date	Isomer Ratio[b]	
	dhATX	dhHTX
14/11/2014	4.7	5.2
21/11/2014	5.2	9.4
30/11/2014	5.3	5.9
04/12/2014	7.0	8.4
10/12/2014	5.6	5.6
16/12/2014	6.8	9.7
23/12/2014	6.9	9.3
01/01/2015	5.8	8.4
07/01/2015	7.3	8.7
13/01/2015	6.3	9.0
20/01/2015	9.4	6.5
07/02/2015	6.5	7.3
19/02/2015	5.9	9.6
28/02/2015	4.0	11.4
Ave	6.2	8.2
SD	1.3	1.8
RSD	21%	22%
Min	4.0	5.2
Max	9.4	11.4

[a] Analysed using the liquid chromatography-mass spectrometry (LC-MS) anatoxin isomers method, where dihydro-anatoxin isomers are assessed underivatised. [b] Isomer ratio was Isomer 1 / Isomer 2, calculated using peak areas. nd = not detected, Ave = Average, SD = Standard deviation, RSD = Relative standard deviation (SD ÷ Ave × 100%), Min = Minimum value, Max = Maximum value.

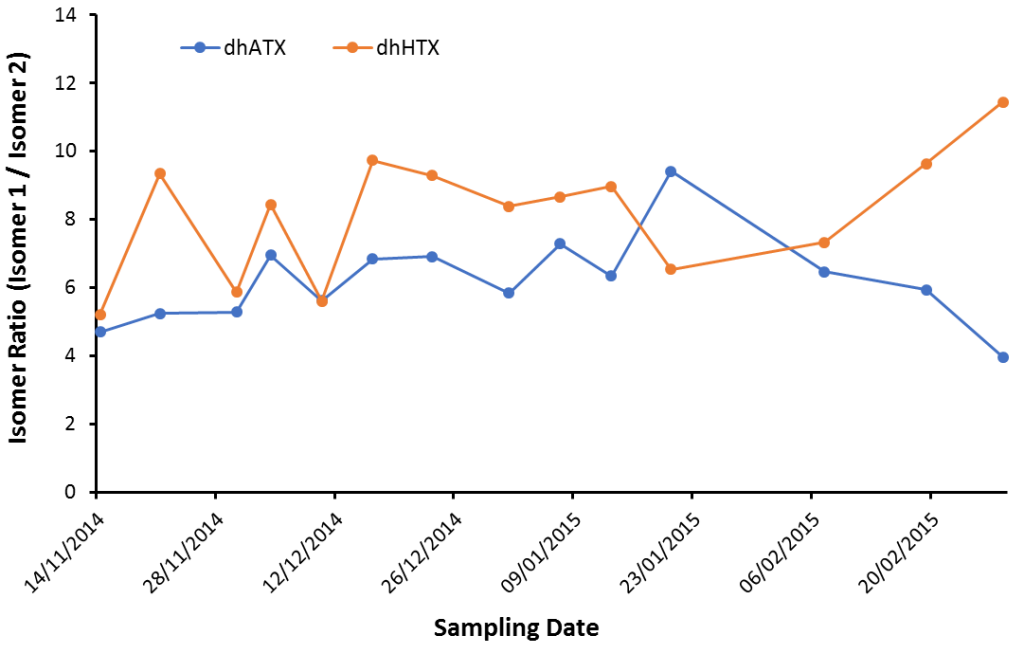

Figure 11: Temporal variation in the isomer composition of dihydro-anatoxin-a and dihydro-homoanatoxin-a (dhATX and dhHTX) in *Phormidium* samples from the Ashley River (Canterbury) using the LC-MS anatoxin isomer method.

To assess whether the dihydro-anatoxin isomer composition varied between *Phormidium* mats under similar conditions (i.e., collected at the same time), a sample set where multiple *Phormidium* mats were collected from a 100 m^2 section of the Hutt River within a 1 hr period was analysed using the LC-MS isomers method. The dhATX and dhHTX isomer composition in the thirteen mat samples collected from the Hutt River were relatively consistent (8% and 13% RSD respectively; Table 8). Whilst there was a high degree of variation from different locations around New Zealand, this data suggests that there may be lower variation of isomer composition in one location at one point in time.

Table 8: Variability of dihydroanatoxin-a and dihydrohomoanatoxin-a (dhATX and dhHTX) isomer compositions in thirteen *Phormidium* mat samples from the Hutt River (Wellington) collected within close proximity.[a]

***Phormidium* Mat**	**Isomer Ratio** [b]	
	dhATX	**dhHTX**
A	5.9	9.5
B	6.9	9.3
C	7.1	11.8
D	6.6	11.2
E	6.4	8.1
F	6.2	12.1
G	6.9	10.3
H	6.6	11.8
I	6.6	8.7
J	6.1	9.5
K	7.1	12.0
L	5.2	10.6
M	6.4	9.8
Ave	6.5	10.4
SD	0.5	1.3
RSD	8%	13%
Min	5.2	8.1
Max	7.1	12.1

[a] Analysed using the liquid chromatography-mass spectrometry (LC-MS) isomers method, where dihydro-anatoxin isomers are assessed underivatised. [b] The isomer ratio was Isomer 1 / Isomer 2, calculated using peak areas. Ave = Average, SD = Standard deviation, RSD = Relative standard deviation (SD ÷ Ave × 100%), Min = Minimum value, Max = Maximum value.

Because of the fluidity observed in the isomer composition of the dihydro-anatoxins, both during their purification and in the natural environment, purified dhATX was freeze-dried and re-dissolved in HCl (0.1 M), acetic acid (0.1% v/v) or water and analysed periodically over 4 days. When the samples were analysed using the LC-MS isomers method, they were either diluted in the solution they were dissolved in or into water. During the 4 day sampling period, no substantial change in isomer composition was observed. However, there were differences associated with the solution the dhATX was dissolved in and the solution it was diluted in for analysis. When dhATX was dissolved in acid it had a different isomer ratio compared to when it was dissolved in water; 5.2 in 0.1 M HCl, 4.9 in 0.1% acetic acid and 4.3 in water (Isomer 1 / Isomer 2; Figure 12). Whilst the difference in isomer composition when dissolved in acidic solutions (0.1 M HCl and 0.1% acetic acid) vs. a neutral solution (water) was statistically significant ($p < 0.05$), it was not substantial from a practical perspective (e.g., to manipulate the isomer composition for toxicology work). When the 0.1 M HCl solution was diluted 1/100 in water prior to LC-MS analysis, there was no change in the dhATX isomer ratio, however, when the 0.1% acetic acid solution was diluted in water the isomer ratio shifted to that observed for dhATX dissolved in water (4.3 Isomer 1 /

Isomer 2). This demonstrates that the dhATX isomer composition can rapidly change depending on the solution it is dissolved in.

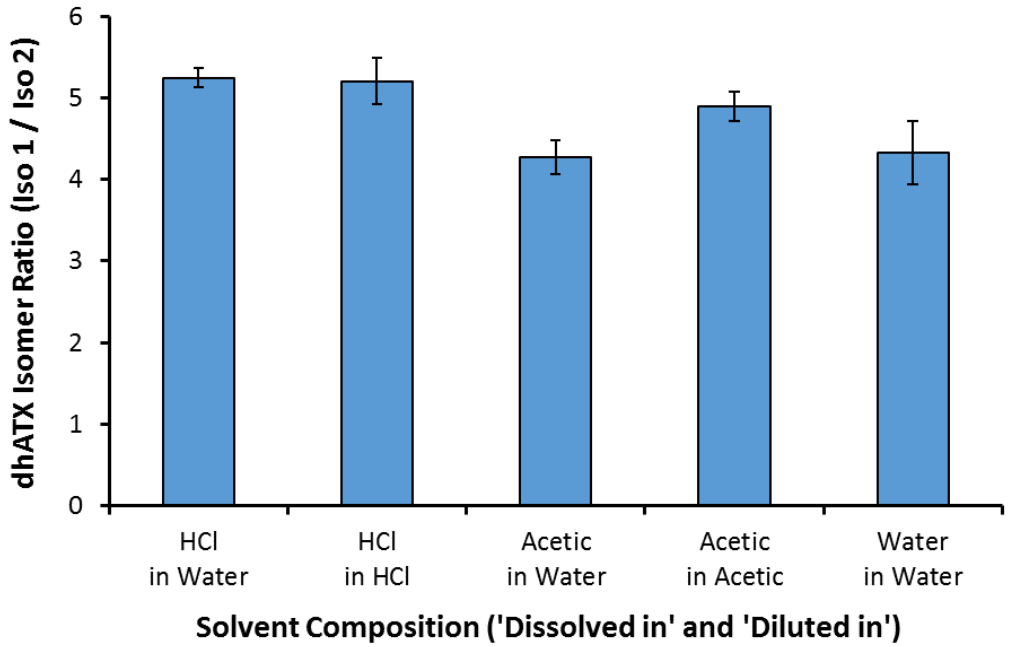

Figure 12: Isomer composition of purified dihydro-anatoxin-a (dhATX) dissolved and then diluted in different solutions, using the LC-MS anatoxin isomer method (HCl = 0.1 M hydrochloric acid, Acetic = 0.1% acetic acid).

3.2.3. NMR Spectroscopy Analysis of Dihydro-Anatoxin Isomers

Whilst assessing the purity of the purified dhATX by NMR, the same sample dissolved in D_2O was re-analysed one week after the initial analysis. When the sample was originally analysed, one day after being dissolved in D_2O, it had an isomer composition of 2.5 Isomer 1 / Isomer 2 (Figure 13A). One week later when the same sample was reanalysed, the isomer composition had shifted to 6.6 Isomer 1 / Isomer 2 (Figure 13B). This exemplifies the LC-MS observations described above, that the dhATX isomer composition is changeable and is susceptible to being altered *ex vivo*. Also, as described in Section 3.1.2, the intensity of the H2 and H10 signals was reduced (Figure 13A vs. Figure 13B). This was due to exchange of the deuterium in the NMR solvent (D_2O) with protons in the dhATX structure during isomerisation. As deuterium is not observed by NMR, the intensity of the signals affected by the deuterium-exchange was lowered.

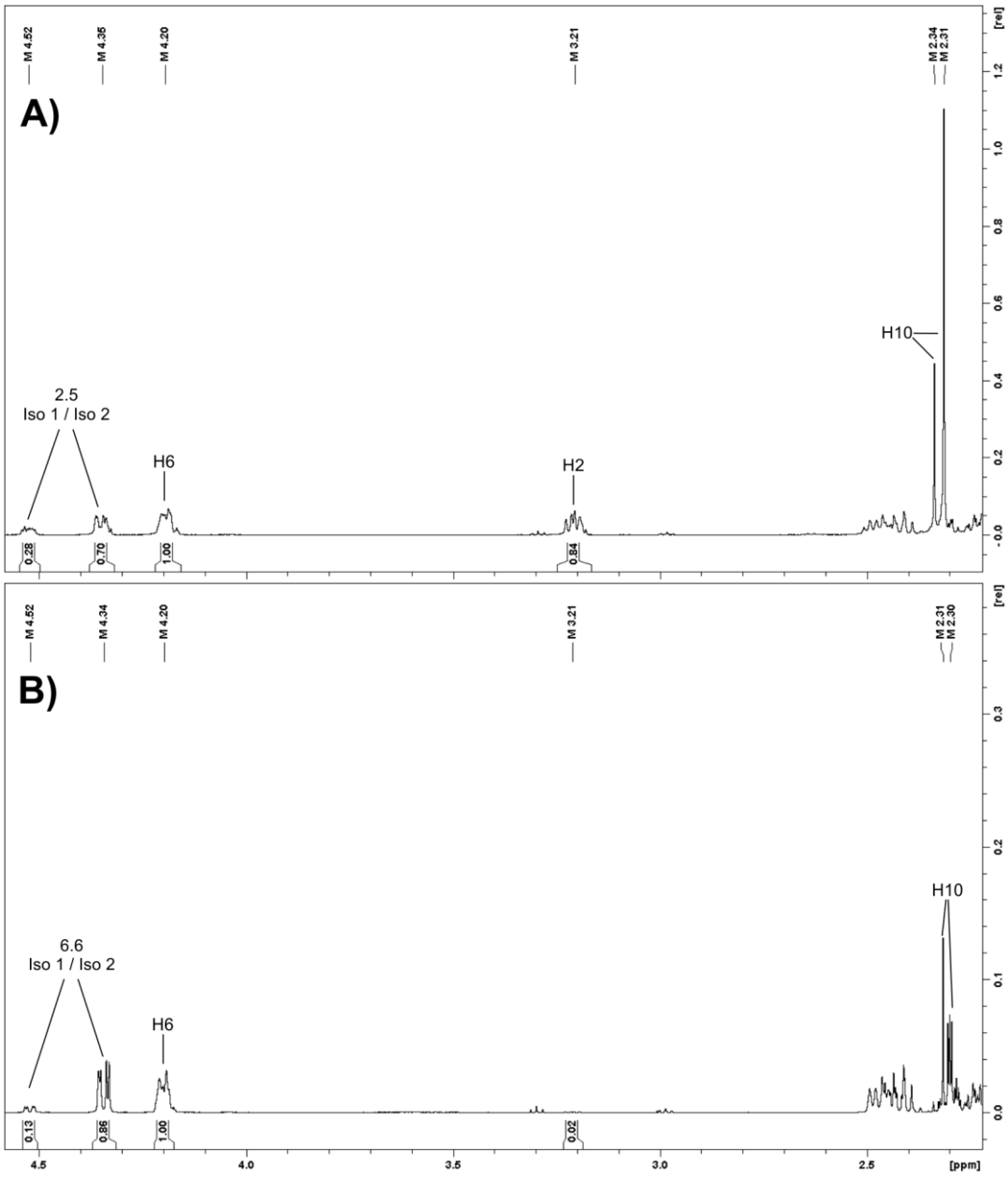

Figure 13: Proton nuclear magnetic resonance (NMR) spectra for a purified dihydroanatoxin-a sample where **A)** the sample was analysed one day after dissolving in D_2O and **B)** seven days later (note: the signal intensity is maintained at approximately the same intensity of the H6 signal).

3.2.4. Summary of the Dihydro-Anatoxin Isomer Composition Work

There was a poor correlation between the isomer ratio for the purified dhATX measured using the LC-MS isomers method (4.3 Isomer 1 / Isomer 2) and the NBD-F derivatisation method (2.2 Isomer 1 / Isomer 2). This might be due to a difference in ionisation efficiency of the two isomers, which is plausible due to the structure of the compound. Because of the movement of dihydro-anatoxin isomer composition observed by LC-MS and NMR, it is difficult to determine which method provides the most accurate measure as procedural

artefacts might be present with each of the methods. Because of this, results from natural *Phormidium* bloom material and the purified dhATX material have only been compared within data sets.

When *Phormidium* samples were assessed for their dihydro-anatoxin isomer composition, there was a range of isomer ratios observed from different rivers around New Zealand. Using the NBD-F derivatisation method, dhATX isomer ratios ranged between 3.7-7.9 Isomer 1 / Isomer 2 in the five rivers assessed (dhHTX isomer ratios were not able to be assessed using this method). The average isomer ratio measured for the purified dhATX material using this method was slightly lower than the lowest level measured in the rivers assessed (2.2 Isomer 1 / Isomer 2). However, because this method displayed poor precision, there was overlap in the individual values observed for the purified dhATX material and the river samples.

Using the LC-MS isomers method to assess *Phormidium* samples from twelve rivers, isomer ratios ranged between 2.9-12.2 Isomer 1 / Isomer 2 for dhATX and 3.9-13.7 Isomer 1 / Isomer 2 for dhHTX. The average isomer ratio measured for the purified dhATX material (dissolved in water) using this method was within the levels observed in the river samples assessed (4.3 Isomer 1 / Isomer 2). When purification of the dhHTX material is complete, the material will be similarly assessed for isomer composition.

The assessment of dihydro-anatoxin isomer composition in *Phormidium* from different New Zealand rivers over the course of a bloom season and within close proximity suggested that drivers such as environmental conditions, *Phormidium* strain and the physical conditions inside the *Phormidium* mats at the time of sampling (e.g., pH) might have the largest influence on the dihydro-anatoxin isomer composition.

The isomer ratio assessed in the purified dhATX material and environmental *Phormidium* bloom material were comparable. Importantly, the dhATX isomer which was always dominant in the environmental material was also the dominant isomer in the purified material. Ideally, the two isomers would be tested separately but with the changeability observed in isomer composition this is not possible without more knowledge on what drives the change in isomer composition. Testing the isomer mixture present in the purified dhATX material will provide new understanding on the relative toxicity of ATX and dhATX, which meets the objective of the project since the isomer composition reflects that seen in the natural environment. We therefore recommend proceeding with the characterisation of the dhATX material in preparation for acute toxicity assessment.

3.3. Acute Toxicity Assessments of Anatoxins in Mice

3.3.1. Acute Toxicity Assessments of Anatoxin-a

The LD_{50} of ATX by intraperitoneal injection was assessed in mice. An LD_{50} of 0.231 mg/kg was obtained, with 95% confidence limits of 0.223-0.250 mg/kg (Table 9). At lethal doses

of ATX, mice became immobile within 2 min after injection and abdominal breathing was noted at this time. Mice remained immobile until shortly before death, when respiratory rates decreased and agitated movements occurred. Death occurred between 10-12 min after injection of ATX. There was no sign of exophthalmia or gasping respiration before death. At sub-lethal doses, immobility and abdominal breathing occurred, but normal activity resumed within 40-45 min. The appearance and behaviour of these mice remained normal throughout the remainder of the day and during the subsequent 13-day observation period. No abnormalities were observed in survivors at necropsy, and the relative weights of major organs were within the normal range. It should be noted that the observed dose-response curve for ATX was remarkably steep; all mice dosed at 0.25 mg/kg died, while mice dosed at 0.223 mg/kg survived.

Table 9: The median lethal dose (LD_{50}) of anatoxin-a (ATX) in mice by intraperitoneal injection, gavage and voluntary consumption.

Route of Administration	**LD_{50} for ATX (mg/kg)** [a]
Intraperitoneal injection	0.231 (0.223-0.250)
Gavage	10.6 (9.9-12.5)
Voluntary consumption [b]	25 (21.81-34)

[a] Figures in brackets indicate 95% confidence limits. [b] By mixing solution with a little ground mouse food and administering over the tongue.

By gavage, the LD_{50} for ATX was 10.6 mg/kg with 95% confidence limits of 9.9-12.5 mg/kg (Table 9). The clinical signs of toxicity were unusual in that animals very quickly became lethargic but then appeared normal for some hours before becoming lethargic again with intension tremors and death. It was thought that this pattern of toxicity could be due to the method of administration; by gavage the solution does not fully mix with the stomach contents and some of the solution can flow around the mass of food to enter the small intestine. This would mean that a portion of the dose could be rapidly absorbed by the small intestine.

The LD_{50} for ATX by voluntary consumption was 25 mg/kg with 95% confidence limits of 21.81-34 mg/kg (Table 9). As observed with the gavage dosing, clinical signs were apparent very quickly, which suggests that this is a genuine response rather than an artefact of dosing by gavage.

3.3.2. Acute Toxicity Assessments of Dihydroanatoxin-a

The LD_{50} of dhATX by ip injection was 0.73 mg/kg with 95% confidence limits of 0.681-0.846 mg/kg (Table 10). The clinical signs and time course of action were consistent with those observed for the ip injection of ATX. The LD_{50} for dhATX by gavage was 2.5 mg/kg with 95% confidence limits of 1.99-3.2 mg/kg. Consistent with that observed during the dosing of ATX by gavage the clinical signs of toxicity were remarkably quick with mice becoming immobile with intension tremor within 10 min post-dosing. At lethal doses, most mice died within 20 min but one mouse initially recovered before dying some hours later. This pattern of toxicity was also observed for ATX. At sub-lethal doses mice became lethargic with intension tremor within 10 min of dosing but showed signs of recovery around 1 h post-dosing. The LD_{50} for dhATX by voluntary consumption was 8 mg/kg with 95% confidence limits of 6.34-10.2 mg/kg. Again, clinical signs of toxicity were observed within 15 min of dosing with mice becoming lethargic and exhibiting intension tremor. At lethal doses animals showed some signs of recovery with death being observed 2.5-7.5 h post-dosing. dhATX was 3.2-times less toxic by voluntary consumption than gavage which is similar to the difference observed for ATX (2.4-times less toxic by voluntary consumption).

Table 10: The median lethal dose (LD_{50}) of dihydro-anatoxin-a (dhATX) in mice by intraperitoneal injection, gavage and voluntary consumption.

Route of Administration	**LD_{50} for dhATX (mg/kg)** [a]
Intraperitoneal injection	0.730 (0.681-0.846)
Gavage	2.5 (1.99-3.22)
Voluntary consumption [b]	8 (6.34-10.2)

[a] Figures in brackets indicate 95% confidence limits. [b] By mixing solution with a little ground mouse food and administering over the tongue.

During Year One of the *Phormidium* Toxicity project, extracts of *Phormidium* rich in dihydro-anatoxins (dhATX and dhHTX) were assessed for acute toxicity in mice,[26] in order to assess the feasibility of conducting toxicology work on purified anatoxin congeners. The only standard available when the *Phormidium* extracts were originally analysed by LC-MS/MS was ATX and this was used to tentatively quantify the concentration of all four anatoxin congeners (ATX, HTX, dhATX and dhHTX). This was not ideal, because the ionisation efficiency of structurally similar compounds can still differ, affecting the analytical results generated. The subsequent qNMR analysis of the purified dhATX (see Section 3.1.2) provided a quantitative standard for this compound and demonstrated that the LC-MS/MS ionisation efficiency (or sensitivity) for ATX was 2-times lower than that of dhATX. This means that the concentrations of dihydro-anatoxins present in the *Phormidium* extracts assessed were 2-times higher than previously thought and, therefore, the LD_{50}

results previously reported are 2-times higher (Table 11). A further caveat of both the original anatoxin analysis and the revised anatoxin analysis is that standards for HTX and dhHTX are not available. Whilst it is assumed that their ionisation efficiency will be similar to ATX and dhATX (respectively) this cannot be confirmed until a standard for each toxin is produced. The revised results presented in Table 11 use the ATX standard to assess the levels of HTX and the dhATX standard to assess the levels of dhHTX.

Table 11: Comparison of the median lethal doses for purified dihydro-anatoxin-a (dhATX) and in a *Phormidium* extract dominated by dihydro-anatoxins, using the original anatoxin analysis[26] and the revised anatoxin analysis using both the ATX standard and the dhATX standard.

Route of Administration	Purified dhATX	*Phormidium* Extract [a]	
		Original Analysis	Revised Analysis
Intraperitoneal injection	0.730 mg/kg	0.377 mg/kg	0.765 mg/kg
Gavage	2.5 mg/kg	4.1 mg/kg	8.3 mg/kg

[a] Where available, the average result of the two toxin-containing *Phormidium* extracts assessed is used.

When the revised anatoxin analysis is used, the ip toxicity for the purified dhATX was comparable to that observed using concentrated extracts of *Phormidium* (Table 11). However, the toxicity results by gavage did not compare well and the average LD_{50} result using the extracts (8.3 mg/kg) was 3.3-times higher than that determined using purified dhATX (2.5 mg/kg). The cause of this difference is difficult to determine without further investigations. It could be related to other components present in the extracts limiting absorption of the toxins. Another possibility is the presence of dhHTX in the extracts although this is an unlikely cause of the change in the toxicity by gavage since dhHTX is only present at 16% relative abundance in the extracts. Finally, it is possible that the dhATX isomer composition in the extracts has influenced the toxicity. At the time that the initial toxicity work was conducted using *Phormidium* extracts, the presence of different isomers of dhATX from *Phormidium* had not been identified. As such, the isomer ratio present in the extracts was not measured until a year later. Preliminary research has shown that the ratio of the two isomers is sensitive to different environmental conditions and conversion is possible, such that the later analysis of the isomer ratios may not reflect that which was actually present when the toxicity testing was undertaken.[28] If one isomer was more toxic than the other, then this could explain the apparent discrepancy between the toxicity of dhATX determined from a pure sample and that from the *Phormidium* extracts.

4. A BIOCHEMICAL TEST FOR MEASURING ANATOXIN-LIKE TOXICITY

A review of relevant literature identified the *Torpedo* (electric eel) electroplaque assay[24,29,30] as a good candidate for assessing anatoxin-like toxicity.[31] *Torpedo* electroplaque makes a

good surrogate for the post-synaptic nAChRs at the mammalian neuromuscular junction (the site of action of anatoxins) as the receptor subunits are very similar.[32] Initial work using the Abraxis *Torpedo* electroplaque kit, indicated that the assay was subject to interferences that might be present in purified anatoxin samples and crude *Phormidium* extracts.[21] This report describes work conducted to assess the assay interferences and to devise strategies to overcome them for purified anatoxin samples. Purified anatoxin samples were then assessed using the *Torpedo* electroplaque assay to determine the nAChR-binding affinity of ATX and dhATX, and how this compared to the acute toxicity results for each congener.

4.1. Mitigating Common Interferences of the *Torpedo* Electroplaque Assay for Assessing Receptor Binding Affinity

Three key elements were established as critical to control whilst analysing samples using the Abraxis *Torpedo* electroplaque biochemical assay:

1) Limit metal contamination by using high-quality water and storing glassware in a biohazard hood to reduce the dust level.
2) Keep the pH close to pH 7 when analysing samples using the assay; samples with acetic acid concentrations < 2.5 mM only need to be diluted two-fold in dilution buffer to reach an acceptable pH.
3) Keep the solution to be assayed approximately isotonic. The optimal range of sodium chloride appears to be 120-190 mM, but it is difficult to be certain without knowing the composition of the kit solutions.

The purified anatoxin samples were neutralised with sodium hydroxide to overcome severe interference (see ‘acetic acid’ in Table 12). Sodium hydroxide was added in small amounts, stepwise, and pH paper was used to monitor pH changes. The acetate salts formed from neutralising the acetic acid were initially expected to provide a sodium chloride concentration close to the optimal range. However, in trials with neutralised acetic acid, the results mimicked that expected with an ionic strength somewhat lower than optimal (Table 12).

Table 12: Assessment of the critical factors for assessing purified anatoxin samples in the Abraxis *Torpedo* electroplaque assay without observing interferences.

Solution	% binding to *Torpedo* nAChRs	
	Blank	ATX Spike [a]
Zero standard	100%	-
Dilution buffer	97%	38%
Water	128%	75%
Acetic acid (HOAc)	12%	17%
Neutralised acetic acid (NaOAc)	120%	47%
NaOAc + salts	105%	39%
125 ppb ATX standard	37%	-

[a] Solution fortified with anatoxin-a at 125 ppb.

The assay protocol suggests using a “ten-fold sample preservation concentrate” with water samples, and it seems that this is to add the required ions (either ionic strength, or possibly specific ions) to the water samples, since a distilled water sample did not give a true result (see ‘water’ result in Table 12). In trials with neutralised acetic acid, addition of 1/10 volume of “sample preservation concentrate” (salts) was found to be a satisfactory treatment to generate accurate ATX results with spiked samples.

4.2. Comparison of the Receptor Binding Affinity Values of Anatoxin Congeners with the Acute Toxicity Values

The purified ATX and dhATX samples were neutralised (to pH 6.5-7) with NaOH and a 1/10 volume of “sample preservation concentrate” was added to each. The final volume was then checked and the final toxin concentration calculated. Both toxins were able to be diluted 100-fold dilution into assay buffer to further limit negative effects. For dhATX, there was no sign of any irregularity being caused by non-toxin components of the solution. For the purified ATX, the assay results indicated that in the year since the ATX sample had been originally assessed, it had either deteriorated or become contaminated with metals. The response was showing as significantly lower than it had previously. An example of results for the kit standards when the buffers became metal-contaminated and the ATX solution retested after one year in storage at -80^{o}C are shown in Figure 14. Metal contamination may have occurred through slow leaching from the vial during the year in storage. Our tentative interpretation is that the acetic acid and formic acid in the vials the ATX was stored in caused leaching of metals from the glass vial over time. The kit manufacturers warn that this assay is very sensitive to metal contamination.

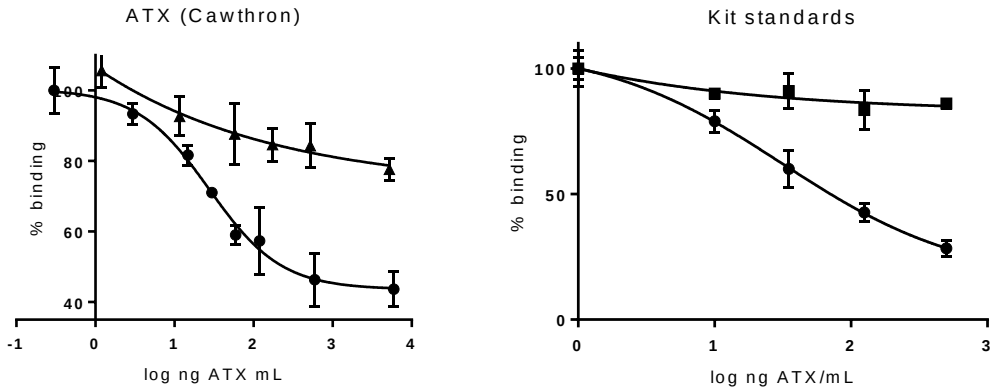

Figure 14: The effects of metal contamination (upper line) on test results for the kit standards, compared with the results of testing the anatoxin-a (ATX) solution before and after a year in storage. In each case the lower line is the expected result.

In order to generate a comparison between the two toxins, it was necessary to use the results from the testing conducted in June 2017.[21] Thus the two toxins, ATX and dhATX have not yet been directly compared, with parallel concentrations, but have instead been independently compared to the ATX standards provided with the kits.

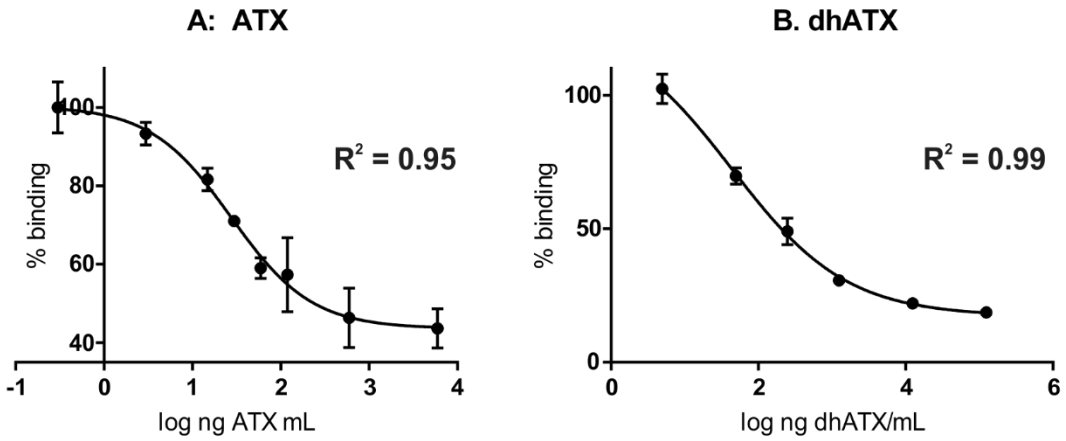

Figure 15: Concentration / response curves for **A)** anatoxin-a (ATX; June 2017) and **B)** dihydroanatoxin-a (dhATX; June 2018) using the Abraxis *Torpedo* electroplaque assay.

Table 13: Assessment of purified anatoxin-a (ATX) and dihydroanatoxin-a (dhATX) using the Abraxis *Torpedo* electroplaque assay, with minor modifications.

	ATX	**dhATX**
IC_{50}	27.5 ng/mL	43.6 ng/mL
Relative toxicity against ATX standards	Equivalent to kit standards (range 0.84 - 1.11-fold, n=3)	3.3-fold less toxic than ATX (range 2.6 - 4.0-fold, n=4)

Both the ATX equivalency results and the IC_{50} results show that dhATX has lower nAChR-binding affinity than ATX (Table 13), although with different multipliers. IC_{50} values are very susceptible to errors induced by not testing over a wide enough range of concentrations. Direct comparison of IC_{50}s can only be performed when results are produced under identical conditions and where the top and bottom plateaus are equivalent, which is not the case here. Thus, the IC_{50} values reported here should not be used to estimate relative toxicities and are indicative only.

The ATX equivalency results determined using the kit standards, however, appear reliable, since they remain fairly constant over a range of different dilutions. These results indicate that dhATX is likely to be somewhat less toxic than ATX, in terms of its ability to bind to the nAChRs at the neuromuscular junction. Because dhATX binds less avidly than ATX, it is likely to be ca. 3-times less toxic to mammals, if nAChR-affinity is the primary driver of toxicity with these toxins.

5. DISCUSSION

Previous toxicology work on ATX is difficult to compare due to differences in the experimental design. No data is available on the toxicity of ATX by voluntary consumption, the most relevant method of administration for anatoxins from *Phormidium* bloom material. Furthermore, the only toxicology work conducted on dhATX used synthetic material with an isomer composition that does not match that observed in the natural environment. During the present study, ATX and dhATX were purified from cyanobacterial source material, assessed for purity by proton NMR spectroscopy and quantified by LC-MS/MS or qNMR respectively. Purifying the toxins from cyanobacterial source material ensured that the structural forms matched that found in the natural environment. Synthetic versions of ATX can sometimes be mixtures of two stereoisomers whilst only (+)-ATX is produced by cyanobacteria. In addition, synthetic versions of dhATX can also contain an unnatural ratio of the C2 isomers as described in Section 3.2. This is also true for semi-synthetic versions of dhATX produced from ATX.

When ATX was assessed for acute toxicity in mice by ip injection, the LD_{50} value established (0.231 mg/kg; 95% confidence limits of 0.223-0.250 mg/kg) was slightly higher than a previous value reported for synthetic (+)-ATX (0.213 mg/kg; 95% confidence limits of 0.197-0.229 mg/kg).[12] Other estimates, again using synthetic material, were significantly higher at 0.316 mg/kg (95% confidence limits of 0.299-0.334 mg/kg)[14] and 0.375 mg/kg,[13] or significantly lower at 0.2 mg/kg.[15] Although with the latter two studies, the form (free base or hydrochloride salt) was unspecified and no confidence limits were reported.

The LD_{50} for ATX determined by gavage (10.6 mg/kg; 95% confidence limits of 9.9-12.5 mg/kg) was slightly lower than a previous determination using synthetic ATX-hydrochloride (LD_{50} of 13.3 mg/kg with 95% confidence limits of 12.6-13.9 mg/kg;

when a conversion to the anatoxin-a free base was made).[12] The acute toxicity when administered by gavage was 46-times lower than when administered by intraperitoneal injection. Previous assessments by Stevens & Krieger (1991)[12] showed a difference of 27-times using an ATX containing extract of *Anabaena flos-aquae* NRC-44-1 and 63-times using synthetic ATX. Our result falls between these two values. To the best of our knowledge, the acute toxicity of ATX has not been reported by voluntary consumption. We determined an LD_{50} of 25 mg/kg for ATX by voluntary consumption, which was 2.4-times less than the LD_{50} by gavage. This difference is comparable with observations for other neurotoxins (e.g., saxitoxin and neosaxitoxin are 2.7- and 1.8-times less toxic by voluntary feeding than by gavage, respectively).[33]

Little research has been performed on the acute toxicity of dhATX, with the data available using synthetic material not matching the isomer composition found in New Zealand *Phormidium*.[15] Whilst the LD_{50} of dhATX by ip injection (0.73 mg/kg) was 3.2-times less toxic than the ip toxicity established for ATX during this project (0.231 mg/kg; Table 14), the oral toxicity for dhATX was higher than ATX. By gavage, the LD_{50} for dhATX was 4.2-times more toxic than ATX (2.5 mg/kg vs. 10.6 mg/kg) and by voluntary consumption, the LD_{50} for dhATX was 3.1-times more toxic than ATX (8 mg/kg vs. 25 mg/kg). These results highlight the importance of conducting toxicology work using methods of administration relevant to the natural situation and demonstrates that ip toxicity is not a good representation of toxicity when the substance is ingested. Since an ip dose does not require absorption of the test compound it is an artificial situation and a correlation between ip toxicity and oral toxicity is often not observed, as absorption and metabolism alter the observed toxicity.

Table 14: Comparison of the median lethal dose (LD_{50}) for anatoxin-a (ATX) and dihydro-anatoxin-a (dhATX) in mice by intraperitoneal injection, gavage and voluntary consumption.

Route of Administration	LD_{50} (mg/kg)		Difference (ATX / dhATX)
	ATX	dhATX	
Intraperitoneal injection	0.231	0.730	0.3
Gavage	10.6	2.5	4.2
Voluntary consumption [a]	25	8	3.1

[a] By mixing solution with a little ground mouse food and administering over the tongue.

Since oral ingestion is the most relevant to human exposure, *Phormidium* blooms that produce dhATX should be treated with caution. As dhATX is often the most prevalent anatoxin congener in New Zealand *Phormidium* blooms,[1] this suggests that there is a potential risk posed by dhATX-producing *Phormidium* in our rivers. However, the severity of this risk is not yet properly understood and risk modelling is required to assess this.

Using the Torpedo electroplaque assay to assess the relative nAChR-binding affinity of ATX and dhATX, ATX was found to be 3.3-times more potent (range of 2.6- to 4-times) than

dhATX. The similarity of this result to the 3.2-fold difference in LD_{50} toxicity for ATX and dhATX by ip injection to mice is striking. For these two toxins, at least, it appears that nAChR-binding affinity is a good surrogate assay for ip toxicity in mice. Despite the ability of this assay to detect these differences, toxicity results conducted *in vivo,* and more specifically by oral administration, are required to provide data for human health risk assessments.

To-date, dhHTX from *Phormidium* bloom material has been purified by multiple chromatography steps including prep-HPLC, but impurities are still present. In its current state, this material is not suitable for toxicity assessment. The impurities either need to be identified and confirmed as benign in comparison to the level of dhHTX present or the impurities need to be removed. As the next purification strategies available involve an increased level of risk of losing the dhHTX, the purification pathway going forward needs to be carefully formulated. Following that, the toxin should be assessed for acute toxicity in mice and for nAChR-binding affinity using the *Torpedo* electroplaque assay.

6. CONCLUSIONS

Two anatoxin congeners found in New Zealand *Phormidium* blooms; ATX and dhATX, were purified from cyanobacteria and were assessed for their acute toxicity and their relative nAChR-binding affinity. The LD_{50} values determined for ATX by ip injection and gavage were comparable to those previously reported and an LD_{50} by voluntary consumption was determined for ATX for the first time. Whilst the acute toxicity for dhATX by ip injection was less toxic than ATX, the acute toxicity for dhATX by gavage and voluntary feeding showed it to be more toxic than ATX by these routes of administration. The difference observed in nAChR-binding affinity for ATX and dhATX paralleled the differences observed in ip toxicity very well, indicating that nAChR-binding affinity might provide a good indication of ip toxicity for anatoxins. However, for the purpose of developing human health risk thresholds, toxicity research needs to be conducted *in vivo,* and more specifically by oral administration since *Phormidium* material is likely to be ingested. Considering the toxicity results observed for dhATX, the feasibility of purifying and assessing the toxicity of dhHTX and HTX should be assessed.

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