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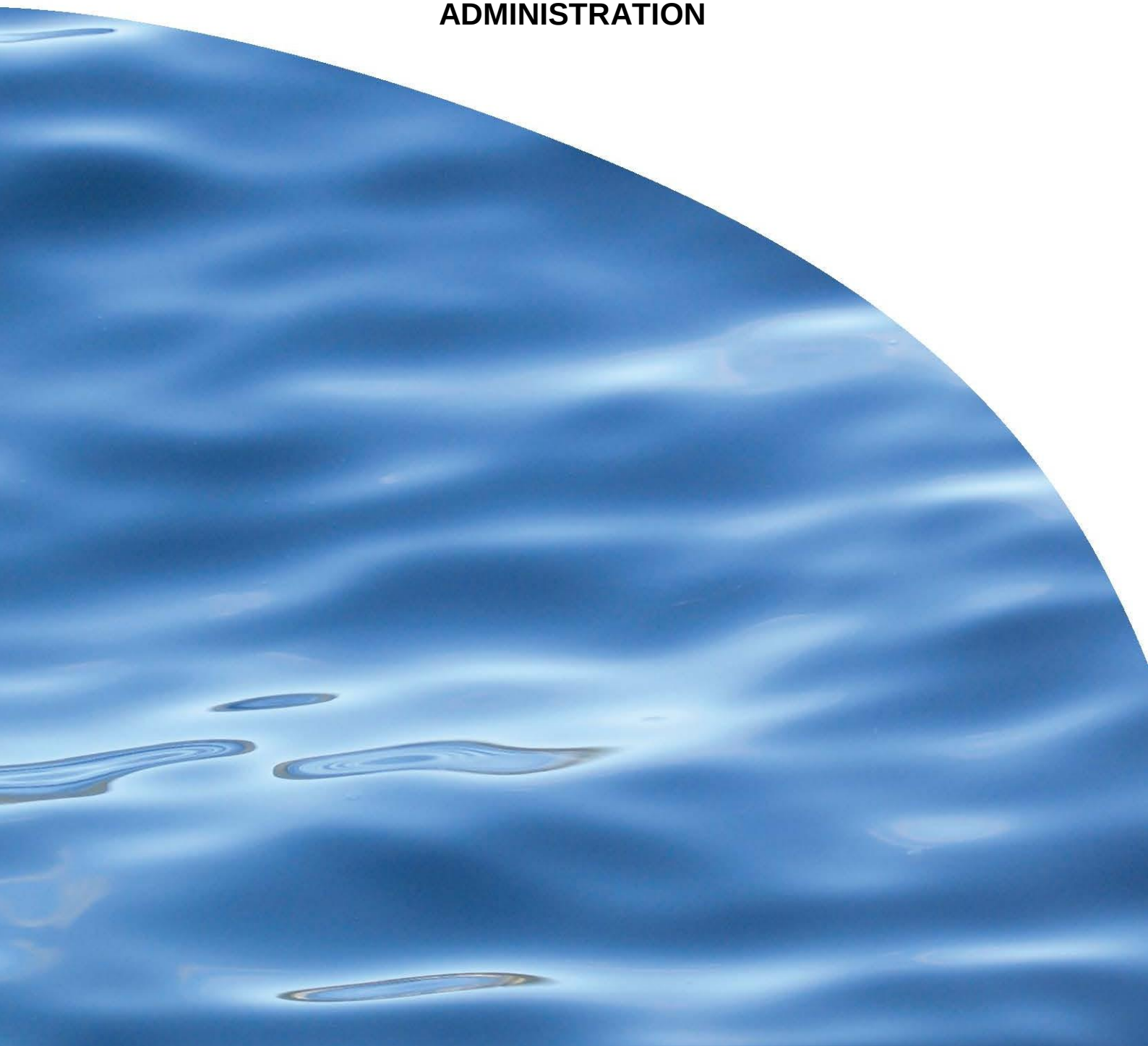
RANGE-FINDING OF ANATOXIN TOXICITY USING *PHORMIDIUM* BLOOM EXTRACTS:

DETERMINING APPROPRIATE STORAGE CONDITIONS AND THE EXPECTED TOXICITY OF ANATOXIN CONGENERS BY THREE ROUTES OF ADMINISTRATION



RANGE-FINDING OF ANATOXIN TOXICITY USING *PHORMIDIUM* BLOOM EXTRACTS:

DETERMINING APPROPRIATE STORAGE CONDITIONS AND THE EXPECTED TOXICITY OF ANATOXIN CONGENERS BY THREE ROUTES OF ADMINISTRATION

Jonathan Puddick,[a] Iain McGrail,[a] Roel van Ginkel,[a]
Susie Wood,[a] Andy Selwood,[a] Rex Munday[b]

[a] Cawthron Institute, Nelson, New Zealand
[b] AgResearch Ruakura, Hamilton, New Zealand

Prepared for the New Zealand Ministry for the Environment

CAWTHRON INSTITUTE
98 Halifax Street East, Nelson 7010 | Private Bag 2, Nelson 7042 | New Zealand
Ph. +64 3 548 2319 | Fax. +64 3 546 9464
www.cawthron.org.nz

REVIEWED BY:
Dr Lesley Rhodes

APPROVED FOR RELEASE BY:
Nico van Loon

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EXECUTIVE SUMMARY

Phormidium is a cyanobacterium which 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 been increasing. *Phormidium* can produce potent neurotoxins called anatoxins and, over the last 15 years, multiple dog deaths have been attributed to dogs consuming the mats. There are inconsistencies and knowledge gaps associated with the toxicological data for the four most prevalent anatoxin congeners; anatoxin-a (ATX), homoanatoxin-a (HTX), dihydro-ATX (dhATX) and dihydro-HTX (dhHTX). To this end, the New Zealand Ministry for the Environment commissioned a study into the toxicity of ATX, HTX, dhATX and dhHTX to mice by three routes of administration; intraperitoneal injection, gavage and voluntary feeding. This report documents the range-finding experiments which were conducted using extracts of *Phormidium* bloom material to determine the feasibility of conducting further toxicity assessments on purified ATX, HTX, dhATX and dhHTX.

Accelerated stability experiments indicated that ATX, HTX and dhHTX are most stable in solutions of pH ≤ 3. A medium-term stability study conducted in 100 mM acetic acid (pH 3) at four temperatures (−70 °C, −20 °C, 4 °C and 18 °C) demonstrated that anatoxins were relatively resistant to degradation at ambient and low temperatures with a rate of −0.01 nM/day (or less) over a five month period. During accelerated stability trials, HTX and dhHTX appeared to be more stable than ATX.

Three extracts of *Phormidium* bloom material were produced and characterised. One extract contained low levels of anatoxins (ALG-02) and the other two extracts contained predominantly dhATX and dhHTX (ALG-01 and ALG-10). The low-anatoxin extract (ALG-02) caused no adverse effects in mice, even at high doses (40,000 mg of extract/kg). The extracts containing dihydro-anatoxins (ALG-01 and ALG-10) caused symptoms similar to those previously-noted for anatoxin poisoning and, at sufficiently high doses, led to death in a similar manner as previously described. When equated to the anatoxin concentration present, a median lethal dose (LD_{50}) of ca. 375 µg/kg (by intraperitoneal injection) was calculated for the dihydro-anatoxins in the extract. This toxicity by intraperitoneal injection is only ca. 1.5-times higher than the LD_{50} reported for ATX, indicating that the dihydro-anatoxin congeners may be more toxic than previously thought. When administered via gavage, the extracts were ca. 11-times less toxic than by intraperitoneal injection. An accurate LD_{50} could not be determined by voluntary feeding at this point in time, as extracts with sufficiently high levels toxin levels could not be produced.

The estimated toxicity of the dihydro-congeners and the observed difference in toxicity between intraperitoneal injection and gavage indicate that sufficient quantities of anatoxin congeners can be purified to undertake the intended toxicology work on purified ATX, HTX, dhATX and dhHTX. The stability results demonstrate that this material can be supplied in a sufficiently-stable form to undertake the toxicity work.

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GLOSSARY

Accelerated stability trials:	A technique used to assess the effect of environmental factors on the stability of a compound within a constrained time period by increasing the rate of degradation, generally by raising the temperature.
ATX:	Anatoxin-a.
Anatoxins:	Collective term to refer to different anatoxin congeners.
dhATX:	Dihydro-anatoxin-a.
dhHTX:	Dihydro-homoanatoxin-a.
DIN:	Dissolved-inorganic nitrogen.
DRP:	Dissolved-reactive phosphorous.
HTX:	Homoanatoxin-a.
HCl:	Hydrochloric acid.
LC-MS/MS:	Liquid chromatography-tandem mass spectrometry.
LD_{50}:	Median lethal dose.
OECD:	Organisation for Economic Co-operation and Development.
PVDF:	Polyvinylidene fluoride.
RSD:	Relative standard deviation.
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 which 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 loads of fine sediment appear to be more susceptible to *Phormidium* blooms.[1]

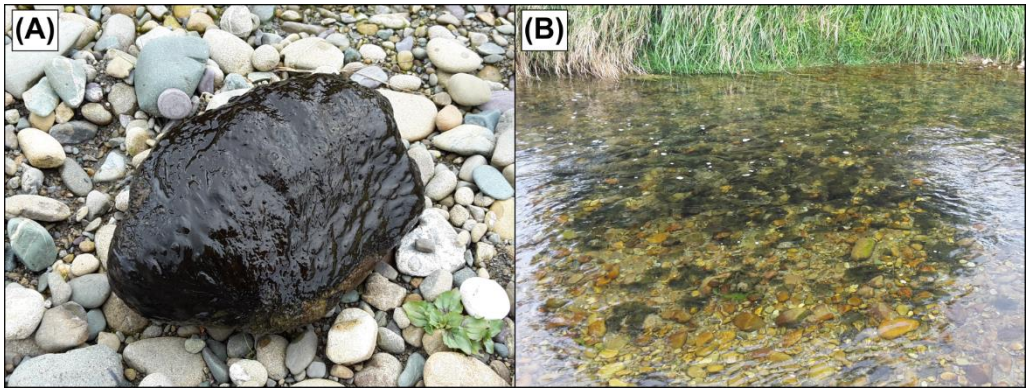
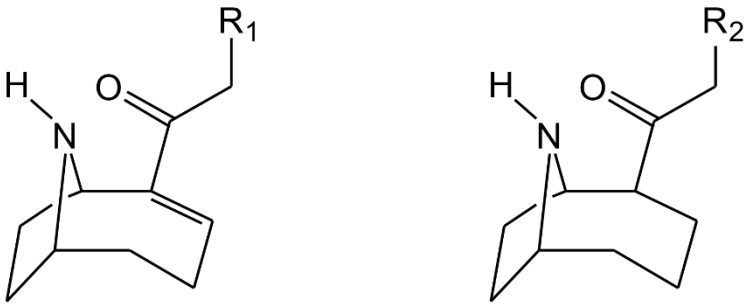


Figure 1: A mat of *Phormidium* growing on a rock **(A)**, and a bloom of *Phormidium* in the Wai-iti River (Tasman region, New Zealand; **B**).

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). These toxins block the nicotinic acetylcholine receptors of the post-synaptic membrane at the neuromuscular junction causing muscular paralysis, leading to death by asphyxia.[7-10] Whilst there is a large body of toxicological data reported for anatoxin-a (ATX; reviewed in Wood et al. 2015),[11] inconsistencies between the experimental methodologies used make reliable interpretation of the results difficult. There is much less data for the other anatoxin congeners prevalent in the *Phormidium* blooms which occur in New Zealand; homoanatoxin-a (HTX), dihydro-ATX (dhATX) and dihydro-HTX (dhHTX; Figure 2). The least information is available on the dihydro-congeners, with the only toxicology information available being performed using a synthetic material which does not match to isomer composition found in the natural environment[12,13] and work performed using an *in vitro* nicotinic acetylcholine binding assay.[14]



ATX R_1 = -H
HTX R_1 = -CH_3
dhATX R_2 = -H
dhHTX R_2 = -CH_3

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

In order to fill these knowledge gaps, the New Zealand Ministry for the Environment commissioned a study into the toxicity of ATX and the anatoxin congeners most prevalent in the *Phormidium* blooms found in New Zealand rivers (HTX, dhATX and dhHTX). To ensure consistency and relevance to the human situation, the toxicity assessments are to be performed according to the guidelines of the Organisation for Economic Cooperation and Development (OECD; Guideline 425, acute oral toxicity)[15] and using three routes of administration; intraperitoneal injection, gavage and voluntary feeding.

During this component of the '*Phormidium* Toxicity' project, range-finding experiments were conducted using extracts of *Phormidium* bloom material to determine the feasibility of conducting further toxicity assessments on purified ATX, HTX, dhATX and dhHTX. These experiments aimed to estimate the difference in toxicity between the dihydro-anatoxin congeners (dhATX and dhHTX) and the anatoxin congeners with more toxicology data available (ATX and HTX). The experiments also aimed to provide an indication of the difference in toxicity observed between the different routes of administration, to determine if sufficient purified material could be produced to conduct the intended work. To ensure the integrity of the samples produced during the course of this study, the stability of anatoxins was assessed to determine a suitable means of storing the *Phormidium* extracts and future purified anatoxin samples.

2. METHODOLOGY

2.1. LC-MS/MS Analysis of Anatoxins

Anatoxin concentrations 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 at on a Thermo Hypersil Gold-aq column (1.9-µm; 50×2.1 mm) at 40 °C using a gradient of water + 0.1% formic acid to acetonitrile

+ 0.1% formic acid at a flow of 0.6 mL/min. Anatoxin quantitation was performed in positive ion mode using multiple reaction monitoring channels for ATX, HTX, dhATX and dhHTX. A certified reference material for ATX (National Research Council, Canada) was used to prepare a five point external calibration curve in 0.1% formic acid (2-100 nM). The concentrations of HTX, dhATX and dhHATX were determined using the ATX certified reference material and a relative response factor of 1. When anatoxin concentrations were outside of the calibration curve, samples were diluted with 0.1% formic acid and re-analysed.

2.2. Stability of Anatoxins

2.2.1. Preparation of Anatoxin Material for Stability Work

A culture of *Cuspidothrix issatschenkoi* (CAWBG02) grown in MLA media[16] at 18 °C under a 12 h:12 h light/dark regime (40 µmol photon/m^2/s) was used as a source for ATX. The culture of CAWBG02 (1,600 mL) was centrifuged to pellet the cells (3,200×g; 10 min). The majority of the supernatant was discarded and the enriched cells (200 mL) were frozen at −20 °C. The ATX was released from the cells by thawing, freezing and thawing again, and the resultant fluid was then clarified by centrifugation (3,200×g; 10 min) and syringe filter (0.45-µm, PVDF; Millipore, USA).

A *Phormidium* bloom sample collected from the Waimea River (Tasman Region) in 2012 was used as a source for HTX, dhATX and dhHTX. *Phormidium* material (2 g) was extracted in water (60 mL) by sonicating for 30 min. The extract was clarified by centrifugation (3,200×g; 10 min) and syringe filter (0.45-µm, PVDF).

The two anatoxin extracts were 'cleaned-up' using a Strata-X C_{18} solid-phase extraction cartridge (1 g; Phenomenex, USA). The cartridge was primed with methanol (50 mL) and equilibrated with water (100 mL) before the samples were loaded. The cartridge was washed with water (50 mL), before the anatoxins were eluted with 10% methanol (200 mL for the CAWBG02 extract and 100 mL for the *Phormidium* extract). Single-use aliquots of each cleaned-up extract were stored at −70 °C until required.

2.2.2. Thermal Stability of Anatoxins

A stock solution of anatoxins was produced by mixing 5 mL of CAWBG02 extract, 5 mL of *Phormidium* extract and 40 mL of water. The final solution was pH 6.6. The solution was aliquoted into amber-coloured glass vials (to protect the solutions from the light), capped and incubated at 55 °C, 70 °C, 90 °C and 105 °C. Samples were removed at 30 min intervals (until 300 min had elapsed), cooled in ice water and immediately analysed by LC-MS/MS to determine the anatoxin concentration. Four samples which were never placed at high temperature were also analysed by LC-MS/MS between 0 min and 30 min. The average of these four results represents the 0 min time-point.

To determine the half-lives at each temperature, first-order rate plots were drawn (i.e., the natural logarithm of the anatoxin concentration was plotted against the incubation time) and the slope / y-intercept of the regression equation was used to calculate the length of time for 50% of each anatoxin congener to degrade.

2.2.3. Stability of Anatoxins at Various pH Values

To evaluate the effect of solution pH on anatoxin stability, an accelerated stability trial was conducted at 90 °C. Hydrochloric acid (HCl), formic acid, acetic acid, phosphate buffer and carbonate buffer (all at a concentration of 100 mM) were used to modify the solution pH (Table 1). Phosphate buffers at each pH were produced by dissolving different ratios of monosodium phosphate and disodium phosphate in water. Carbonate buffers at each pH were produced using 100 mM solutions of sodium bicarbonate and sodium carbonate mixed at different ratios. The pH of each modifier solution was confirmed using a pH meter (Orion Star A211; Thermo Scientific, USA).

Table 1: Composition of pH modifiers used for anatoxin stability experiment.

Solution pH	pH Modifier [a]
1.4	Hydrochloric acid
2.5	Formic acid
3.0	Acetic acid
5.2	Phosphate buffer
6.1	Phosphate buffer
7.1	Phosphate buffer
8.1	Phosphate buffer
8.9	Phosphate buffer
10.4	Carbonate buffer
11.2	Carbonate buffer

[a] All modifiers were at a concentration of 100 mM.

In a walk-in fridge (4 °C), stock anatoxin solutions at each pH were produced by mixing 4 mL of CAWBG02 extract, 4 mL of *Phormidium* extract and 32 mL of pH modifier. The pH of each solution was measured after preparation and did not change by more than 0.1 pH. The solutions were aliquoted into amber vials, capped and incubated at 90 °C. At various time-points up to 240 min (4 h), duplicate samples were removed from the 90 °C oven and immediately placed at −70 °C to limit further degradation. Samples were raised to ambient temperature and analysed by LC-MS/MS within 2 h to determine the anatoxin concentration. Samples were maintained at 4 °C in the LC-MS/MS auto-sampler. Two samples at each pH, which were not exposed to high temperature, were analysed at the start of the experiment to act as the 0 min time-point. Half-lives were determined as described in Section 2.2.2.

2.2.4. Medium-Term Stability of Anatoxins Solutions at pH 3

A stock solution of anatoxins was produced by mixing 20 mL of CAWBG02 extract, 20 mL of *Phormidium* extract and 160 mL of 100 mM acetic acid. The final solution was pH 3. The solution was aliquoted into amber vials, capped and stored at −70 °C, −20 °C, 4 °C and 18 °C. At periodic time-points over five months, triplicate samples from each storage temperature were removed, bought to ambient temperature and analysed by LC-MS/MS to determine the anatoxin concentration. Sufficient aliquots were prepared so that the samples could be discarded following analysis. Triplicate samples were analysed immediately after preparation of the stock solution and represented the 0 day time-point for each temperature.

2.3. Preparation of *Phormidium* Extracts for Toxicity Assessment

2.3.1. Collection and Assessment of Phormidium Bloom Material

Phormidium bloom samples were collected from rivers around the Tasman, Canterbury and Wellington regions. Samples were collected by scraping the *Phormidium* mats from rocks into a net using gloved hands. The *Phormidium* was transferred into buckets and stored at −20 °C.

The *Phormidium* material was defrosted at 4 °C after being supplemented with 0.1% acetic acid (final concentration). Once defrosted (3-9 days; depending on the sample size), the environmental material was divided into freeze-drying trays, re-frozen at −20 °C, and then chilled to −70 °C. The samples were freeze-dried at 0.2 mBar vacuum, where the shelf temperature was gradually raised from −20 °C to 15 °C over the course of five days (Gamma 1-16 LSC freeze-drier; Martin Christ Gefriertrocknungsanlagen, Germany). The trays of freeze-dried *Phormidium* were transported in a sealed container to a pre-cleaned, fume-hood for milling. In the fume-hood, the dried material was broken up into a coarse powder (1-15 mm pieces) and milled in a blender, in 500 g batches. The milled powder was stored in airtight containers at −20 °C until extraction and analysis.

Each milled *Phormidium* bloom sample was mixed thoroughly and four subsamples (1 g each) were weighed into 50 mL Falcon tubes. Each sample was extracted in 0.1% acetic acid (20 mL) by mixing on a vortex mixer for 30 s, sonicating for 30 min, freezing at −20 °C and thawing in a sonicator bath for a further 30 min. Extracts were clarified by centrifugation (3,200×g; 10 min).

To estimate the level of dissolved solids in the extract, an aliquot of the extract (10 mL) was transferred to pre-weighed 50 mL Falcon tube which had been conditioned on the freeze-dryer overnight. This was frozen at −70 °C, freeze-dried and re-weighed. To determine the anatoxin content of the *Phormidium* material, the extract was analysed by LC-MS/MS as described in Section 2.1.

2.3.2. Preparation and Characterisation of Phormidium Bloom Extracts

Three extracts of *Phormidium* bloom samples (ALG-01, ALG-02 and ALG-10) were prepared for toxicity assessment. Milled *Phormidium* material (50 g; Section 2.3.1) was blended in 500 mL of 0.1% acetic acid for 1-2 min. The extract was centrifuged (3,200×g; 10 min) and vacuum-filtered through Whatman's No. 1 filter paper. The clarified extract was dried on a rotary evaporator to concentrate the extract to ca. 50 mL. This concentrated extract was frozen at −70 °C, freeze-dried and stored at −20 °C until required. The dried extract was ground to a fine powder using a metal spatula and weighed into a 250 mL Duran bottle (ca. 4 g) and 100 mM acetic acid was added to re-suspend the extract at 200 mg/mL (ca. 20 mL). This was resuspended by sonicating for 30 min, freezing then thawing. As not all of the extract components re-dissolved at the high extract concentrations, the samples were clarified by centrifugation (3,200×g; 10 min), vacuum-filtration (GF/F; Whatman, UK) and syringe-filtration through a 0.45-µm filter, then a 0.22-µm filter (PVDF; Millipore, USA).

To determine the amount of dissolved solids, an aliquot of each extract (0.2 mL) was transferred to a weighed 50 mL Falcon tube, which had been pre-conditioned on the freeze-dryer overnight. The extract was frozen at −70 °C, freeze-dried and re-weighed. To determine the anatoxin concentration of the extracts, triplicate aliquots of each extract were analysed by LC-MS/MS. Each aliquot was initially diluted 1/10 in 0.1% formic acid, centrifuged at 17,000×g for 5 min and serially diluted to 1/100, 1/1,000, or 1/10,000 in 0.1% formic acid according to the expected anatoxin concentration.

At the conclusion of the toxicity work, the remainder of the *Phormidium* extracts were returned to Cawthron for analysis of anatoxin concentration by LC-MS/MS. Each aliquot was initially diluted 1/10 in 0.1% formic acid, centrifuged at 17,000×g for 5 min and serially diluted to 1/100, 1/1,000, or 1/10,000 in 0.1% formic acid. Only the ALG-10 extract was re-tested, as the entirety of the ALG-01 extract was used to complete the toxicity work and the ALG-02 extract contained low levels of anatoxins.

2.4. Toxicological Evaluation

The acute toxicities of the *Phormidium* extracts were determined according to the principles of Organisation for Economic Co-operation and Development (OECD) Guideline 425,[15] and median lethal doses (LD_{50}s) were calculated using the computer programme associated with this guideline.[17] The animal experiments were approved by the Ruakura Animal Ethics Committee. In the case of the ALG-01 extract, appropriate aliquots of the sample were diluted with 1 mM HCl for dosing. With the other extracts, it was necessary to freeze-dry aliquots of the samples, after which the solid material was taken up into 1 mM HCl for dosing. The volume of solution administered by intraperitoneal 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 and food intakes 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, small intestine, caecum and large intestine of all the mice were recorded at necropsy, and relative organ weights were calculated as a percentage of body weight.

3. RESULTS AND DISCUSSION

3.1. Stability of Anatoxin Congeners

As the integrity of the toxicity results from this study rely on the administration of a known quantity of anatoxin to mice, the stability of anatoxins was assessed. This brief stability study aimed to determine a suitable solution for the storage of anatoxins prior to toxicological assessment and to assess the medium-term stability of anatoxins in this solution.

3.1.1. Thermal Stability of Anatoxin Congeners at pH 6.6

In order to determine an appropriate temperature to conduct accelerated stability experiments on anatoxins, the thermal stability of anatoxins at temperatures ≥ 55 °C was assessed. A mixture of anatoxins (ATX, HTX, dhATX and dhHTX; at pH 6.6) were incubated at 55 °C, 70 °C, 90 °C and 105 °C for various lengths of time. At 55 °C, anatoxin degradation was negligible over the 300 min time-course of the experiment (Figure 3A). At 70 °C, ATX and dhHTX concentrations decreased during the time-course of the experiment (Figure 3B). At 90 °C and 105 °C, more rapid degradation was observed, with concentrations of all anatoxin congeners decreasing to low levels within 90 min (Figure 3C and Figure 3D).

At times, the concentration of dhATX increased prior to decreasing (Figure 3A-C). As this trend was observed in subsequent experiments, it is likely that some dhATX was being formed from an unidentified precursor. The source of the unidentified precursor was likely to be the *Cuspidothrix issatschenkoi* culture (CAWBG02; formerly *Aphanizomenon*), as this cyanobacterium has been shown to produce an 11-carboxyl biosynthetic precursor for ATX.[18] Since it was not possible to distinguish between the dhATX starting material and the dhATX formed during the experiments, the data for dhATX was not used for further analyses.

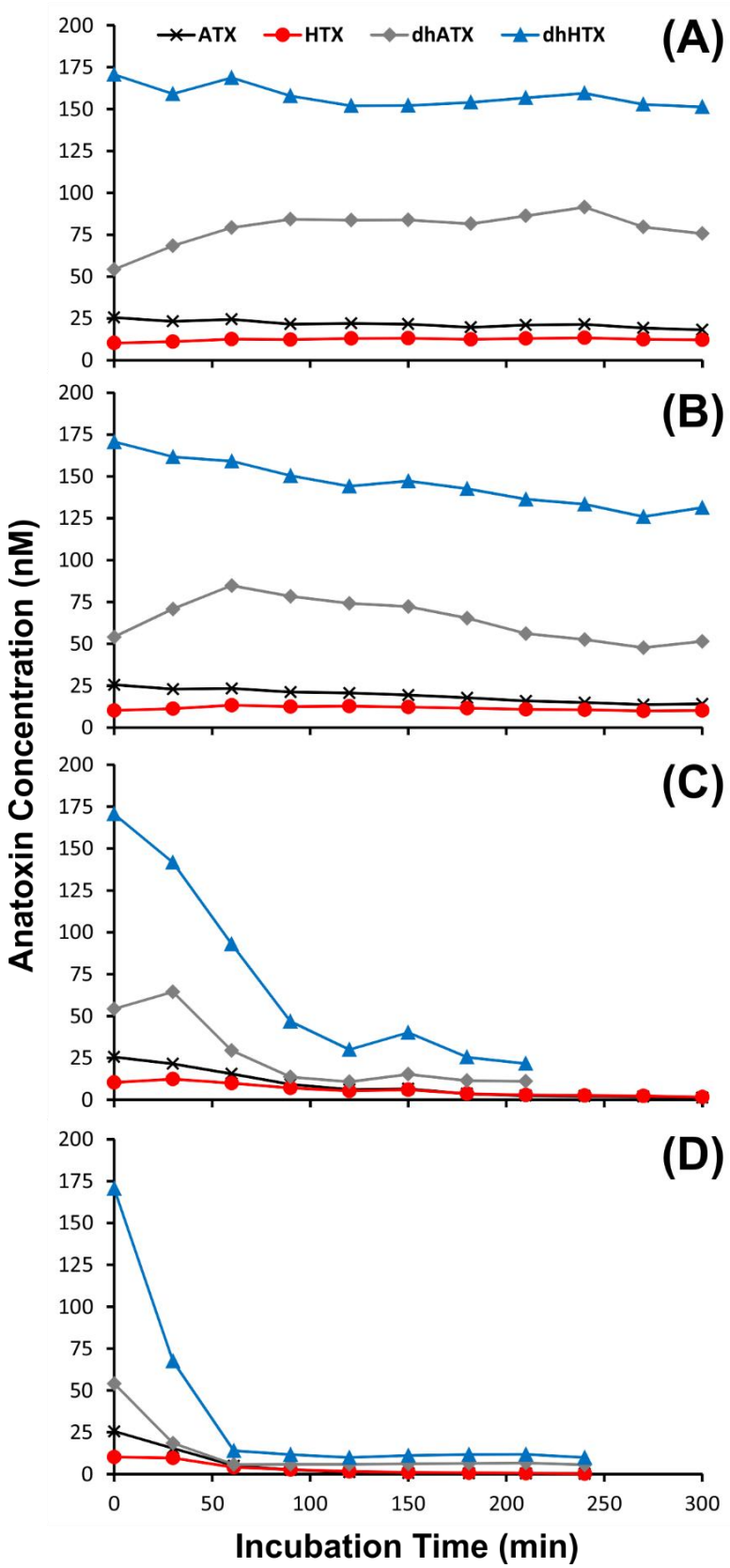


Figure 3: Time-course of anatoxin degradation at temperatures of **A)** 55 °C, **B)** 70 °C, **C)** 90 °C and **D)** 105 °C (ATX = anatoxin-a, HTX = homoanatoxin-a, dhATX = dihydro-ATX, dhHTX = dihydro-HTX).

As expected, the half-lives for ATX, HTX and dhHTX decreased with higher temperatures, as thermal instability increased (Table 2). Half-lives for HTX were not able to be calculated at the lower temperatures of 55 °C and 70 °C, as no decrease in concentration was observed during the time-course of the experiment. In general, HTX and dhHTX appeared to be more stable than ATX (Table 2).

Table 2: Half-lives for anatoxin-a (ATX), homoanatoxin-a (HTX) and dihydro-HTX (dhHTX) at temperatures 55 °C and above.

Temperature	Half-life (min)		
	ATX	HTX	dhHTX
55 °C	1,600	-	6,380
70 °C	770	-	2,560
90 °C	160	210	240
105 °C	65	85	80

Half-lives for HTX at 55 °C and 70 °C were not able to be determined as a decrease in concentration was not observed during the time-courses of those experiments.

3.1.2. Accelerated Stability Assessment of ATX, HTX and dhHTX at Various pH Values

Accelerated stability trials are used to assess the effects of different environmental factors on the stability of a compound within a constrained time period. Generally, the temperature of the reaction is raised so that the compound degrades more quickly. Using the half-lives determined in Section 3.1.1, a temperature of 90 °C was chosen to assess the effect of altering the solution pH on the stability of ATX, HTX and dhHTX. This temperature was selected as the compounds degrade slowly enough to collect samples at multiple time-points and quickly enough that the experiment could be conducted within a single day.

A range of pH values between pH 1.4 and pH 11.2 were assessed and the half-lives at 90 °C were determined (Table 3). As observed with the thermal stability assessment (Table 2), HTX and dhHTX consistently had higher half-lives than ATX (Table 3), indicating that they are more stable than ATX under these conditions. A general trend observed for ATX, HTX and dhHTX was lower stability in higher pH solutions (more alkaline) and higher stability in lower pH solutions (more acidic; Figure 4). A plateau in stability is potentially reached around pH 3, as the stability of ATX and dhHTX was relatively consistent at pH ≤ 3. As half-lives for HTX could not be determined at pH 1.4 and pH 2.5, this plateau in stability could not be confirmed with the HTX data. These accelerated stability results indicate that anatoxins are most stable in solutions with a pH ≤ 3.

In the past, stability work has only been conducted on ATX, not HTX and dhHTX. The results of those studies also demonstrated that ATX was more stable at lower pH.[19,20] The range and resolution of solution pHs used in the previous work were restrictive (three or four solution pHs), and the data presented here provides a good picture of how adversely ATX, HTX and dhHTX are affected by high pH.

Table 3: Half-lives for anatoxin-a (ATX), homoanatoxin-a (HTX) and dihydro-HTX (dhHTX) at a temperature of 90 °C and a range of pH values.

pH	Half-life (min)		
	ATX	HTX	dhHTX
1.4	2,100	-	-
2.5	1,870	-	8,610
3.0	2,110	57,940	8,600
5.2	980	20,650	5,160
6.1	650	1,440	4,290
7.1	240	300	1,850
8.1	110	130	330
8.9	80	90	150
10.4	55	70	100
11.2	45	55	85

Several half-lives for HTX and dhHTX were not able to be determined as a decrease in concentration was not observed during the time-courses of those experiments.

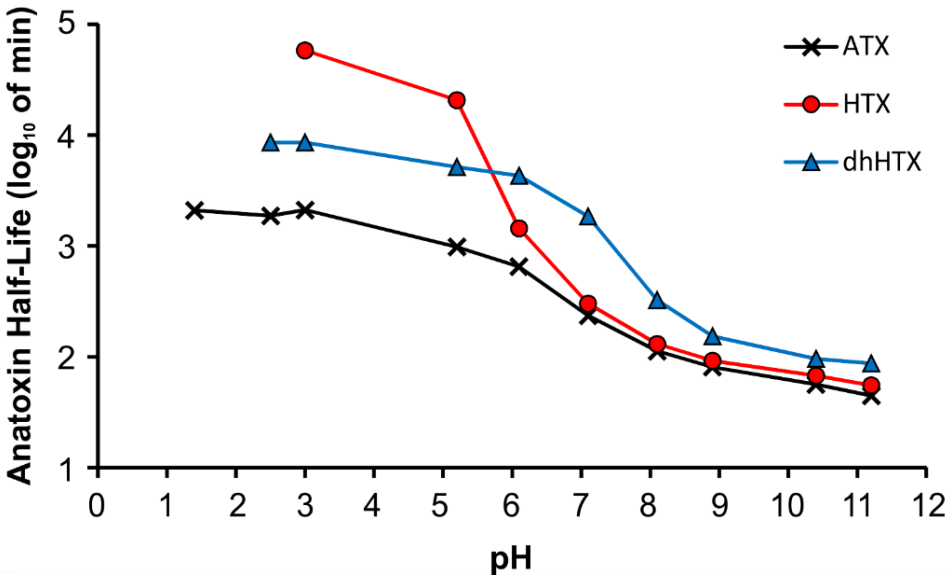


Figure 4: Plot of anatoxin half-lives over a range of pH values (note: half-lives are $\log_{10}$ adjusted; ATX = anatoxin-a, HTX = homoanatoxin-a, dhHTX = dihydro-HTX).

3.1.3. Medium-Term Stability of ATX and HTX at Acidic pH

To assess the stability of ATX, HTX and dhHTX during medium-term storage (several months), anatoxins were dissolved in acetic acid (100 mM; pH 3) and stored at −70 °C, −20 °C, 4 °C and 18 °C. Samples were periodically removed from storage and analysed for anatoxin concentration over a five month period (155 days). Random fluctuations in the anatoxin concentrations (ATX, HTX and dhHTX) were observed over this period (Figure 5).

The level of intermediate precision encountered (≤ 8.9% relative standard deviation; RSD) was expected for an LC-MS/MS method for anatoxin analysis (up to 10% RSD).

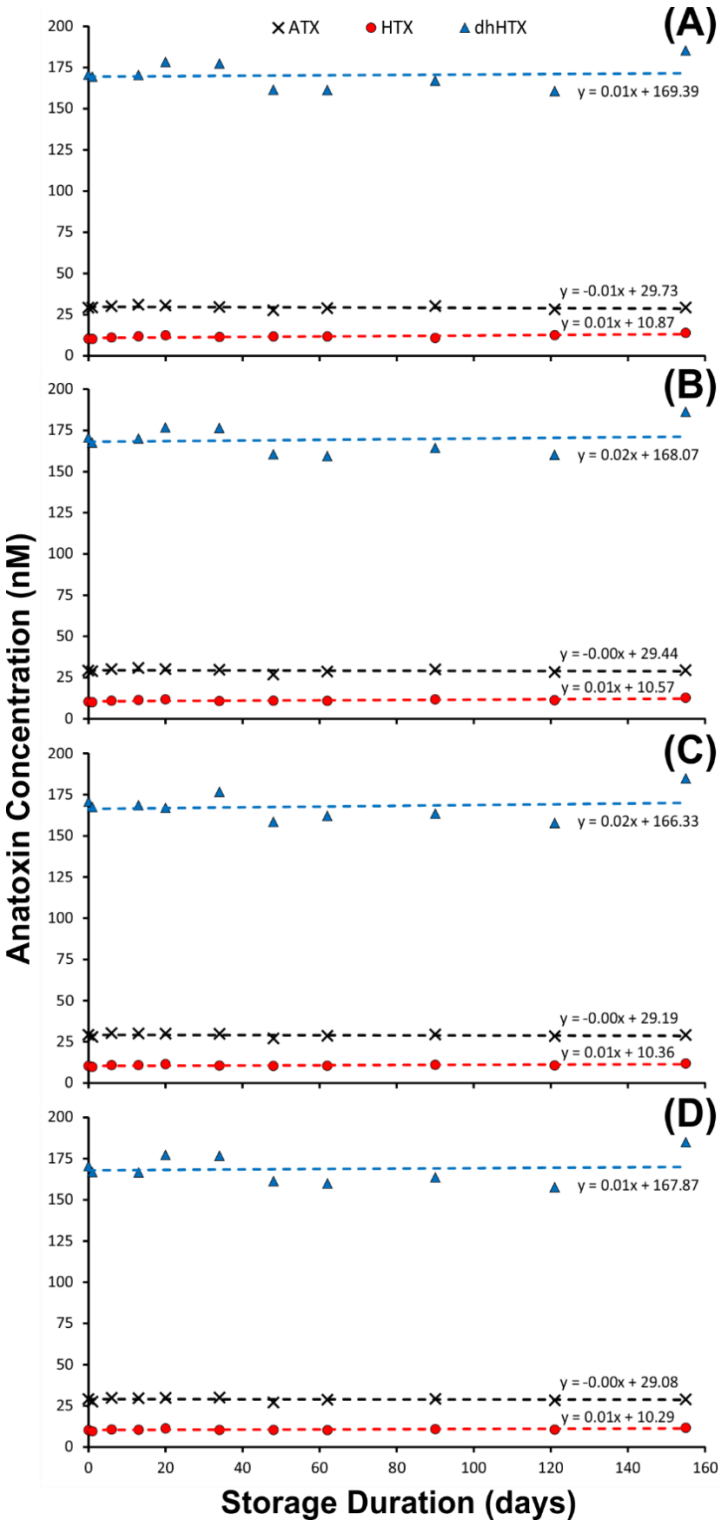


Figure 5: Medium-term stability trial for anatoxin-a (ATX), homoanatoxin-a (HTX) and dihydro-HTX (dhHTX) at temperatures of **A)** 18 °C, **B)** 4 °C, **C)** −20 °C and **D)** −70 °C (data points are the average of triplicate samples).

No trends in decreasing concentration were observed for ATX, HTX or dhHTX, with regression slopes for each anatoxin congener remaining close to zero (ranging from −0.01 nM/day to +0.02 nM/day; Figure 5). Consistent trends in the stability of ATX, HTX and dhHTX were observed at each of the four storage temperatures (Figure 5A-D). In the graph it may appear that the precision for the dhHTX measurements were greater than the other anatoxin congeners (Figure 5), however the average RSD for dhHTX (across all temperatures) was lower than that of HTX (5.1% RSD for dhHTX and 6.4% RSD for HTX). The fluctuation appears more substantial for dhHTX because of the higher concentrations of dhHTX present in the anatoxin mixture.

Overall, the results from this medium-term stability trial demonstrate that ATX, HTX and dhHTX dissolved in 100 mM acetic acid are stable for sufficient time to assess their effects in toxicity assays, without compromising the accuracy of the anatoxin quantitation performed. As ATX, HTX and dhHTX maintained at 4 °C and 18 °C showed similar stability to anatoxins maintained at −70 °C, it is not essential to send anatoxin solutions on dry ice to maintain their integrity. Shipping samples overnight with wet ice will be sufficient to maintain the temperature at < 18 °C.

3.2. Preparation of *Phormidium* Extracts for Toxicity Assessment

3.2.1. Phormidium Bloom Material Collections

Seven *Phormidium* bloom samples were collected from rivers of the Tasman, Canterbury and Wellington regions during the months of March and April 2016. Two archived samples collected in 2011 and 2012 were also assessed. They ranged in size from 2-33 kg (wet weight) or 0.1-2.4 kg when dried (Table 4).

Table 4: Samples collected to assess the toxicity of *Phormidium* bloom material.

Sample ID	Collection Site	Region	Date	Wet Weight	Dry Weight
ALG-01	Waimea River (by Appleby Bridge)	Tasman	Dec 2012	3 kg	0.2 kg
ALG-02	Opihi River (at SH1)	Canterbury	24/03/16	6 kg	0.4 kg
ALG-03	Opihi River (at SH1)	Canterbury	24/03/16	10 kg	0.5 kg
ALG-04	Ashburton River	Canterbury	26/04/16	6 kg	0.6 kg
ALG-05	Temuka River	Canterbury	28/04/16	2 kg	0.3 kg
ALG-06	Big Rock (Silverstream)	Wellington	31/03/16	13 kg	0.8 kg
ALG-07	Wai-iti River (by Waimea West Rd)	Tasman	16/03/16	20 kg	0.8 kg
ALG-09	Silverstream	Wellington	10/03/16	2 kg	0.1 kg
ALG-10	Hutt River	Wellington	2011	33 kg	2.4 kg

3.2.2. Anatoxin Content of Phormidium Bloom Material

The nine *Phormidium* bloom samples were dried and milled, before being assessed for their anatoxin content. The total anatoxin content of the dried *Phormidium* material ranged from 4-160,000 µg/kg (of dried material; Table 5). The ALG-01 and ALG-10 samples had the highest anatoxin content, whilst ALG-07 had the lowest concentration. The only samples with yields of total anatoxins >1 mg were the ALG-01, ALG-05 and ALG-10 samples. Combined, these three samples contain 1.3 mg of HTX, 350 mg of dhATX and 55 mg of dhHTX.

Table 5: Anatoxin contents of the *Phormidium* bloom samples.

Sample ID	Anatoxin Content (µg of toxin/kg of dried material)				Anatoxin Yield (mg of anatoxins)
	ATX	HTX	dhATX	dhHTX	
ALG-01	1,600	5,300	77,800	73,000	31
ALG-02	8	ND	12	3	< 0.1
ALG-03	8	ND	8	1	< 0.1
ALG-04	4	ND	13	2	< 0.1
ALG-05	ND	ND	33,100	3,600	13
ALG-06	ND	6	32	2	< 0.1
ALG-07	ND	3	1	ND	< 0.1
ALG-09	13	340	510	420	0.1
ALG-10	16	120	135,000	16,700	364

ND = Below the limit of detection (< 1 µg of toxin/kg of dried material), ATX = anatoxin-a, HTX = homoanatoxin-a, dhATX = dihydro-ATX, dhHTX = dihydro-HTX.

When the anatoxin content of the *Phormidium* bloom samples was expressed as the amount of toxin present in the dried extract (as opposed to the dried mats), the highest anatoxin contents were observed in the ALG-01, ALG-05 and ALG-10 samples (Table 6). The lowest anatoxin contents was observed in ALG-07 (0.03 µg/g of extract), and ALG-02, ALG-03 and ALG-04 had a similarly low anatoxin content (0.12-0.16 µg/g of extract).

Table 6: Extract yields and anatoxin contents of 0.1% acetic acid extracts of the *Phormidium* bloom samples.

Sample ID	Extract Yield (g of extract/kg of dried material)	Anatoxin Content (µg of toxin/g of extract)				
		ATX	HTX	dhATX	dhHTX	Total
ALG-01	92	17	58	850	800	1,700
ALG-02	170	0.05	ND	0.07	0.02	0.13
ALG-03	140	0.05	ND	0.06	0.01	0.12
ALG-04	130	0.03	ND	0.11	0.02	0.16
ALG-05	150	ND	ND	230	25	260
ALG-06	84	ND	0.07	0.38	0.02	0.47
ALG-07	180	ND	0.02	0.01	ND	0.03
ALG-09	170	0.08	2.0	3.0	2.4	7.5
ALG-10	230	0.07	0.54	580	72	650

ND = Below the limit of detection (< 0.01 µg of toxin/g of extract), ATX = anatoxin-a, HTX = homoanatoxin-a, dhATX = dihydro-ATX, dhHTX = dihydro-HTX.

Three samples were selected for preparation of larger-scale extracts for toxicity assessment. Ideally these three samples would contain; **1)** predominantly HTX, **2)** predominantly dihydro-anatoxin congeners (dhATX and dhHTX) and **3)** low quantities of anatoxins. None of the samples contained predominantly HTX, however, ALG-01 contained the highest anatoxin content and HTX was present in the sample (Table 6). ALG-10 was also selected as it contained high concentrations of dhATX and dhHTX (Table 6). The ALG-07 sample was originally selected for preparation as it contained the lowest anatoxin content (Table 6). However, during the production of the ALG-07 extract, several complications were encountered. Whilst the pigmentation of the ALG-01 and ALG-10 was blue, the ALG-07 extract was brown coloured. Also, the extract yield of the ALG-07 material was lower than anticipated. For these reasons, an extract of ALG-02 was produced for the toxicity assessment instead, as it also contained similarly low levels of anatoxins (0.13 µg/g of ALG-02 extract vs. 0.03 µg/g of ALG-07 extract; Table 6). The ALG-02 extract also had a more substantial extract yield and blue pigmentation.

3.2.3. Characterisation of Phormidium Bloom Extracts for Toxicity Assessment

Larger-scale extracts of the three *Phormidium* samples selected for toxicity assessment (ALG-01, ALG-02 and ALG-10) were prepared and assessed for their anatoxin content and level of dissolved solids. All three of the extracts had similar colouration (deep blue) and a similar level of dissolved solids (118-130 mg/mL; Table 7). The anatoxin concentration in ALG-01 was the highest (420 µg/mL; Table 7 and Appendix 1), followed by ALG-10 (100 µg/mL), and the anatoxin concentration of the ALG-02 extract was >4,000-times lower (0.023 µg/mL).

Table 7: Characterisation of the *Phormidium* extracts prepared for toxicity assessment.

Extract	Colour	Volume (mL)	Dissolved Solids (mg/mL)	Anatoxin Concentration (µg/mL)				
				ATX	HTX	dhATX	dhHTX	Total
ALG-01	Blue	6	125	0.3	3	360	60	420
ALG-02	Blue	15	118	0.008	ND	0.015	ND	0.023
ALG-10	Blue	12	130	ND	0.6	90	10	100

ND = Below the limit of quantitation (< 0.0005 µg/mL), ATX = anatoxin-a, HTX = homoanatoxin-a, dhATX = dihydro-ATX, dhHTX = dihydro-HTX.

The anatoxin composition of the ALG-01 and ALG-10 extracts was mostly dihydro-anatoxin congeners (dhATX and dhHTX; Figure 6) with smaller amounts of HTX (1% in each). The ALG-01 extract also contained trace levels of ATX (0.1%). The ratio of dhATX todhHTX in the ALG-01 extract was not as expected, as the earlier analysis of the *Phormidium* material had a ratio of ca. 1:1 dhATX/dhHTX (Table 6). The 6:1 ratio (dhATX/dhHTX) observed in the ALG-01 extract prepared for toxicity assessment was likely due to slightly different solubility for the two compounds (in 100 mM acetic acid) in conjunction with the high dissolved solids concentration of the extract. This may have limited the solubility of the dhHTX as the solution approached saturation.

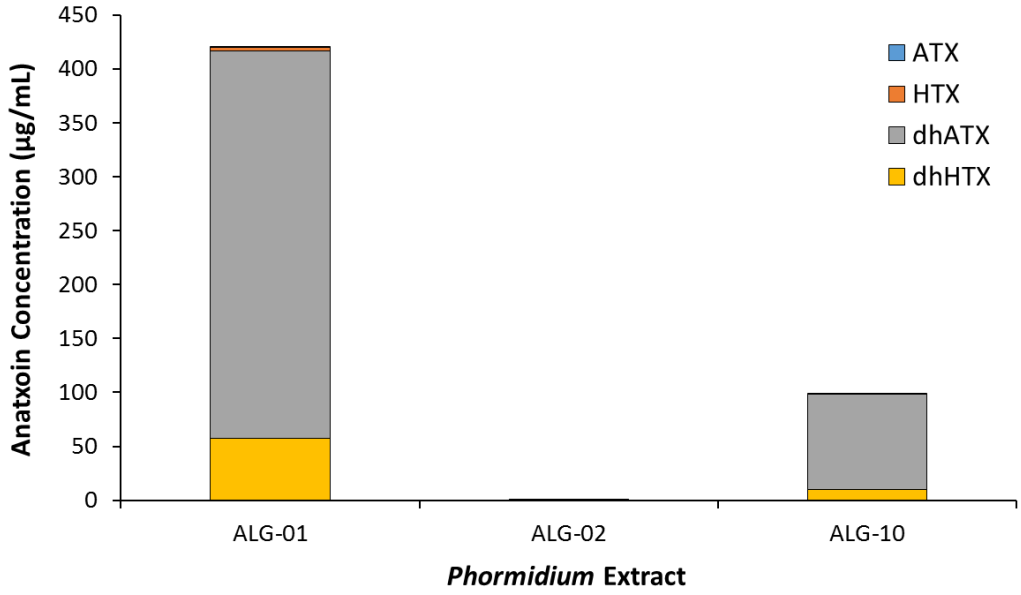


Figure 6: Anatoxin concentration and composition of the three *Phormidium* extracts produced for toxicity assessment (ATX = anatoxin-a, HTX = homoanatoxin-a, dhATX = dihydro-ATX, dhHTX = dihydro-HTX).

Following toxicity assessment, the remainder of the ALG-10 sample was returned to Cawthron to reassess the anatoxin concentration. Between the initial testing conducted in September 2016 until the retest conducted in January 2017, there was a 2% decrease in total anatoxin concentration for the ALG-10 extract (Table 8 and Appendix 2). From an analytical perspective, the observed change in anatoxin concentration was minimal and

within the expected levels of variability for LC-MS/MS testing. Regardless, the close agreement in anatoxin concentrations demonstrates that the extract was not subject to severe degradation during transport or storage as a part of conducting the toxicology work. Unfortunately, the ALG-01 extract submitted for toxicity assessment could not be re-analysed as the entirety of the sample was used to conduct the toxicology work. The ALG-02 extract was not assessed due to its low anatoxin levels (Table 7).

Table 8: Anatoxin concentrations of the ALG-10 *Phormidium* extract prior to toxicity assessment and following toxicity assessment.

Testing Date	Anatoxin Concentration (µg/mL)				
	ATX	HTX	dhATX	dhHTX	Total
Pre-Toxicity Work (September 2016)	ND	0.6	87.8	10.4	98.8
Post-Toxicity Work (January 2017)	ND	0.6	85.7	10.2	96.4

ND = Below the limit of quantitation (< 0.0005 µg/mL), ATX = anatoxin-a, HTX = homoanatoxin-a, dhATX = dihydro-ATX, dhHTX = dihydro-HTX. Note: the ALG-01 extract could not be re-analysed as the entirety of the sample was used to conduct the toxicology work and the ALG-02 extract was not assessed due to its low level of anatoxins.

3.3. Toxicological Evaluation of Three *Phormidium* Extracts

We planned to estimate the LD_{50}s of the three *Phormidium* extracts to mice through administration by intraperitoneal injection, gavage and voluntary consumption. However, because of the lower toxicity of the ALG-10 extract, estimation of toxicity by voluntary consumption was not possible. Furthermore, the toxicity of ALG-02 was such that acute toxic effects could not be induced by any route of administration, even at high levels of dosing.

3.3.1. Toxicity of ALG-01

Toxicity of ALG-01 by intraperitoneal injection

The LD_{50} for the ALG-01 extract by intraperitoneal injection was 820 mg/kg, with 95% confidence limits between 795 and 890 mg/kg. At lethal doses, mice became immobile soon after dosing, with abdominal breathing. The respiration rates of the animals decreased, and cyanosis and exophthalmia were observed shortly before death, which occurred between 12-18 min after dosing. At sub-lethal doses, mice became very lethargic, with abdominal breathing. The mice became more active 60-90 min after dosing, although periods of lethargy were observed throughout the day. After 24 h, their appearance and behaviour were normal, and remained so during the remainder of the 14-day observation period. They continued to gain weight during this period, and food intakes were within the normal range. No macroscopic changes were recorded at necropsy, and relative organ weights were normal.

Toxicity of the ALG-01 Extract by Gavage

The LD_{50} of the ALG-01 extract by gavage was 10,000 mg/kg, with 95% confidence limits between 8,300 and 11,500 mg/kg. The symptoms of intoxication were the same as those recorded after intraperitoneal injection, and, at very high doses, death again occurred within minutes. At doses close to the LD_{50}, however, death occurred up to 8 h after dosing. Again, recovery occurred at sub-lethal doses, with normal weight gain and food intake. No abnormalities were recorded at necropsy, and organ weights were within the normal range.

Toxicity of the ALG-01 Extract by Voluntary Consumption

For experiments on toxicity by voluntary consumption, mice are trained to eat small quantities of cream cheese. The test material is mixed with the cream cheese and the mixture is provided to the mice, which is then rapidly consumed. From past experience, the acute toxicity of materials administered by voluntary consumption is generally 2-3 times lower than that by gavage. The LD_{50} of the ALG-01 extract by gavage was 10,000 mg/kg, so a starting point for feeding studies would be 2.5 times this (i.e., 25,000 mg/kg, a dose of 500 mg for a 20 g mouse). This weight of ALG-01 extract was freeze-dried and the dry material was mixed with cream cheese. However, mice trained to eat cream cheese refused to eat the mixture, so a different approach was required. ALG-01 extract (500 mg) was freeze-dried and mixed with a small amount of finely-powdered mouse food. This was delivered on to the top of the tongue of a 20 g mouse, using a positive displacement pipette with a glass capillary. The mixture was immediately chewed and swallowed by the mouse, but no toxic effects were observed. A higher dose was tested using this technique, giving the dry material from 800 mg of ALG-01 extract, thereby providing a dose of 40,000 mg/kg. Again, no toxic effects were observed. It was not possible to give a dose higher than this, so we must conclude that the acutely toxic dose of ALG-01 by voluntary consumption is more than four times higher than that by gavage.

3.3.2. Toxicity of the ALG-10 Extract

Toxicity of the ALG-10 Extract by Intraperitoneal Injection

The LD_{50} of the ALG-10 extract by intraperitoneal injection was 4,100 mg/kg, with 95% confidence limits between 3,950 and 4,450 mg/kg. The symptoms of intoxication induced by this extract were the same as those induced by the ALG-01 extract, and death was again rapid at lethal doses, with death times between 11-13 min. Survivors recovered completely, and no abnormalities or changes in organ weight were recorded at necropsy.

Toxicity of the ALG-10 Extract by Gavage

The LD_{50} of the ALG-10 extract by gavage was 39,500 mg/kg, with 95% confidence limits between 33,400 and 45,400 mg/kg. Again, the symptoms of intoxication were the same as those observed with the ALG-01 extract, and survivors recovered completely.

Toxicity of the ALG-10 Extract by Voluntary Consumption

In view of the low toxicity of ALG-10 by gavage, it was not practicable to determine an LD_{50} for this extract by voluntary consumption.

3.3.3. Toxicity of the ALG-02 Extract

Toxicity of the ALG-02 Extract by Intraperitoneal Injection

No deaths occurred after an intraperitoneal dose of 40,000 mg/kg of the ALG-02 extract. Dosing at higher levels was not practicable. The LD_{50} of this extract by intraperitoneal injection is therefore >40,000 mg/kg. The mice did not exhibit any adverse symptoms upon dosing, and no abnormalities were observed in the organs.

Toxicity of the ALG-02 Extract by Gavage and Voluntary Consumption

Because of the low toxicity of this extract, determination of toxicity by oral administration and voluntary feeding was not practicable.

3.3.4. Discussion of the Toxicity Results

Previously, ATX has been shown to be a potent agonist of the nicotinic acetylcholine receptor, leading to blockade of neuromuscular transmission. At lethal doses, death is due to muscle paralysis, leading to death by respiratory failure.[21] The symptoms of intoxication observed in this study with the *Phormidium* extracts, which contained a mixture of HTX, dhATX and dhHTX, were consistent with the same mechanism of action. After adjusting for the different concentrations of total anatoxins in the ALG-01 and ALG-10 extracts (Table 7), the LD_{50}s of these extracts were similar; 344 and 410 µg/kg respectively by intraperitoneal injection and 4,200 and 3,950 µg/kg by gavage.

Table 9: Median lethal doses for the three *Phormidium* extracts expressed per mg of extract administered and according to the amount of total anatoxins present in the extract (*in brackets*).

***Phormidium* Extract**	**Intraperitoneal Injection**	**Gavage**	**Voluntary Feeding**
ALG-01	820 mg extract/kg (*344 µg anatoxins/kg*)	10,000 mg extract/kg (*4,200 µg anatoxins/kg*)	>40,000 mg extract/kg
ALG-02	>40,000 mg extract/kg	NA	NA
ALG-10	4,100 mg extract/kg (*410 µg anatoxins/kg*)	39,500 mg extract/kg (*3,950 µg anatoxins/kg*)	NA

NA = Not able to be assessed due to low sample toxicity.

Since the *Phormidium* extract containing low levels of anatoxins (ALG-02; essentially a blank for the other components in the extracts) demonstrated no toxicity for mice (Table 9), the toxicity observed with the ALG-01 and ALG-10 extracts was not due to other compounds present in the *Phormidium* extracts. Therefore, the toxicity data for the ALG-01 and ALG-10 extracts may be compared to the acute toxicities reported for ATX. Median lethal doses of 320 µg/kg,[22] 200 µg/kg[12] and 260 µg/kg[23] have been reported for synthetic (+)-ATX by intraperitoneal injection. The data from the present study indicates that the dihydro-anatoxin

congeners, dominant in the ALG-01 and ALG-10 *Phormidium* extracts, could have a LD_{50} closer to ATX than previously thought (ca. 375 µg/kg by intraperitoneal injection). This was unexpected, as synthetic dhATX hydrochloride was reported to be much less toxic to mice, with an intraperitoneal LD_{50} of approximately 2,500 µg/kg,[12] a finding which is consistent with the observation that synthetic dhATX was approximately 10-times less inhibitory at the nicotinic receptor than anatoxin-a.[14] It should be noted, however, that the isomeric composition of natural dhATX is markedly different from that of the synthetic material,[13] and the stereochemistry of anatoxin derivatives has a pronounced influence on toxicity, as shown by the much lower toxicity of synthetic (−)-ATX compared with the natural (+)-ATX isomer.[22]

Previous oral toxicity work involving administration by gavage has produced LD_{50}s of 13,300 µg/kg using synthetic (+)-ATX and 6,700 µg/kg using ATX extracted from *Anabaena flos-aquae* NRC-44-1.[23] Compared to the corresponding LD_{50} results by intraperitoneal injection, conducted during that study (250 µg/kg and 260 µg/kg),[23] a difference in toxicity of 27-63 times was observed. During the present study, we observed a relatively consistent difference in toxicity between administration via intraperitoneal injection and gavage, with LD_{50}s by gavage being ca.11-times higher. This value is consistent with data reported for HTX extracted from *Oscillatoria formosa* where a 10-fold difference in toxicity was observed between the two methods of administration.[24] Between the two dosing regimens, the times till death were also different. With intraperitoneal injection, all deaths occurred within 20 min after dosing. Whilst similar death times were seen at high oral doses of the *Phormidium* extracts, doses closer to the LD_{50} resulted in death at up to 8 h after dosing. These differences in time till death are common, as injected materials are rapidly absorbed from the peritoneum and absorption from the gut is slower.

During the present study an accurate assessment of the difference in toxicity between administration via gavage and voluntary feeding was not possible, as producing a sufficiently-concentrated anatoxin solutions was not achievable. When purified anatoxin congeners are used in future studies, this problem will be alleviated as co-extractives will not be present.

4. CONCLUSIONS AND RECOMMENDATIONS

During this study we demonstrated that ATX, HTX and dhHTX are most stable in solutions with a pH ≤ 3 using an accelerated stability trial. Our results also indicated that HTX and dhHTX may be more stable than ATX. During a medium-term stability trial (five months) using anatoxins dissolved in 100 mM acetic acid, no degradation or only minimal degradation (−0.01 nM/day) was observed for ATX, HTX and dhHTX. No difference in medium-term stability was observed between the different temperatures assessed (−70 °C, −20 °C, 4 °C and 18 °C). The medium-term stability results demonstrate that anatoxins dissolved in 100 mM acetic acid are stable for sufficient time to conduct accurate toxicology

assessments. The stability of dhATX was not able to be directly assessed during this study, as the compound appears to be formed from an unidentified precursor present in our extract.

In order to estimate the difference in toxicity for anatoxins between administration via intraperitoneal injection, gavage and voluntary feeding, and to gain some insight into the likely toxicity of the dihydro-anatoxin congeners (dhATX and dhHTX), three *Phormidium* extracts were prepared for toxicity assessment by mouse bioassay. Two of these extracts contained predominantly dihydro-anatoxin congeners and one extract contained only very low levels of anatoxins. Our toxicology work on the extracts indicated that administration of anatoxins to mice by gavage is ca. 11-times less toxic than administration by intraperitoneal injection. An accurate estimate of the difference in toxicity expected via voluntary feeding was not possible during this study, but should be possible using purified anatoxin congeners. Toxicity assessment of the extracts also demonstrated that the dihydro-anatoxin congeners produced by *Phormidium* are likely to be more toxic than previously thought. The dihydro-anatoxin congeners appear to be ca. 1.5-times less toxic than ATX, as opposed to being 10-times less toxic as indicated by other research. Accurate toxicology data using purified dhATX and dhHTX is still required, but the higher than expected toxicity means that purification of sufficient dhATX and dhHTX from environmental material is a viable option.

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6. APPENDICES

Appendix 1: Anatoxin analysis of the ALG-01, ALG-02 and ALG-10 *Phormidium* extracts prior to toxicity assessment (September 2016).

Extract	Anatoxin Concentration (µg/mL)					Total Anatoxin (µg/mL)		
	ATX	HTX	dhATX	dhHTX	Total	Ave	SD	RSD
ALG-01	0.4	3.2	360.8	57.0	421.2	420.3	0.9	0.2%
	0.3	3.2	358.1	58.0	419.6			
	0.3	3.2	359.2	57.2	420.0			
ALG-02	0.008	ND	0.015	ND	0.023	0.0236	0.0002	1.1%
	0.008	ND	0.015	ND	0.024			
	0.008	ND	0.016	ND	0.024			
ALG-10	ND	0.6	87.5	10.2	98.2	98.8	0.5	0.5%
	ND	0.6	88.0	10.4	99.0			
	ND	0.6	88.0	10.5	99.0			

ND = Below the limit of quantitation (< 0.0005 µg/mL), ATX = anatoxin-a, HTX = homoanatoxin-a, dhATX = dihydro-ATX, dhHTX = dihydro-HTX, Ave = average, SD = standard deviation, RSD = relative standard deviation.

Appendix 2: Anatoxin analysis of the ALG-10 *Phormidium* extract following toxicity assessment (January 2017).

Extract	Anatoxin Concentration (µg/mL)					Total Anatoxin (µg/mL)		
	ATX	HTX	dhATX	dhHTX	Total	Ave	SD	RSD
ALG-10	ND	0.6	85.7	10.0	96.3	96.4	0.5	0.5%
	ND	0.6	85.1	10.2	96.0			
	ND	0.6	86.2	10.2	97.0			

ND = Below the limit of quantitation (< 0.0005 µg/mL), ATX = anatoxin-a, HTX = homoanatoxin-a, dhATX = dihydro-ATX, dhHTX = dihydro-HTX, Ave = average, SD = standard deviation, RSD = relative standard deviation. Note: the ALG-01 extract could not be re-analysed as the entirety of the sample was used to conduct the toxicology work and the ALG-02 extract was not assessed due to its low level of anatoxins.