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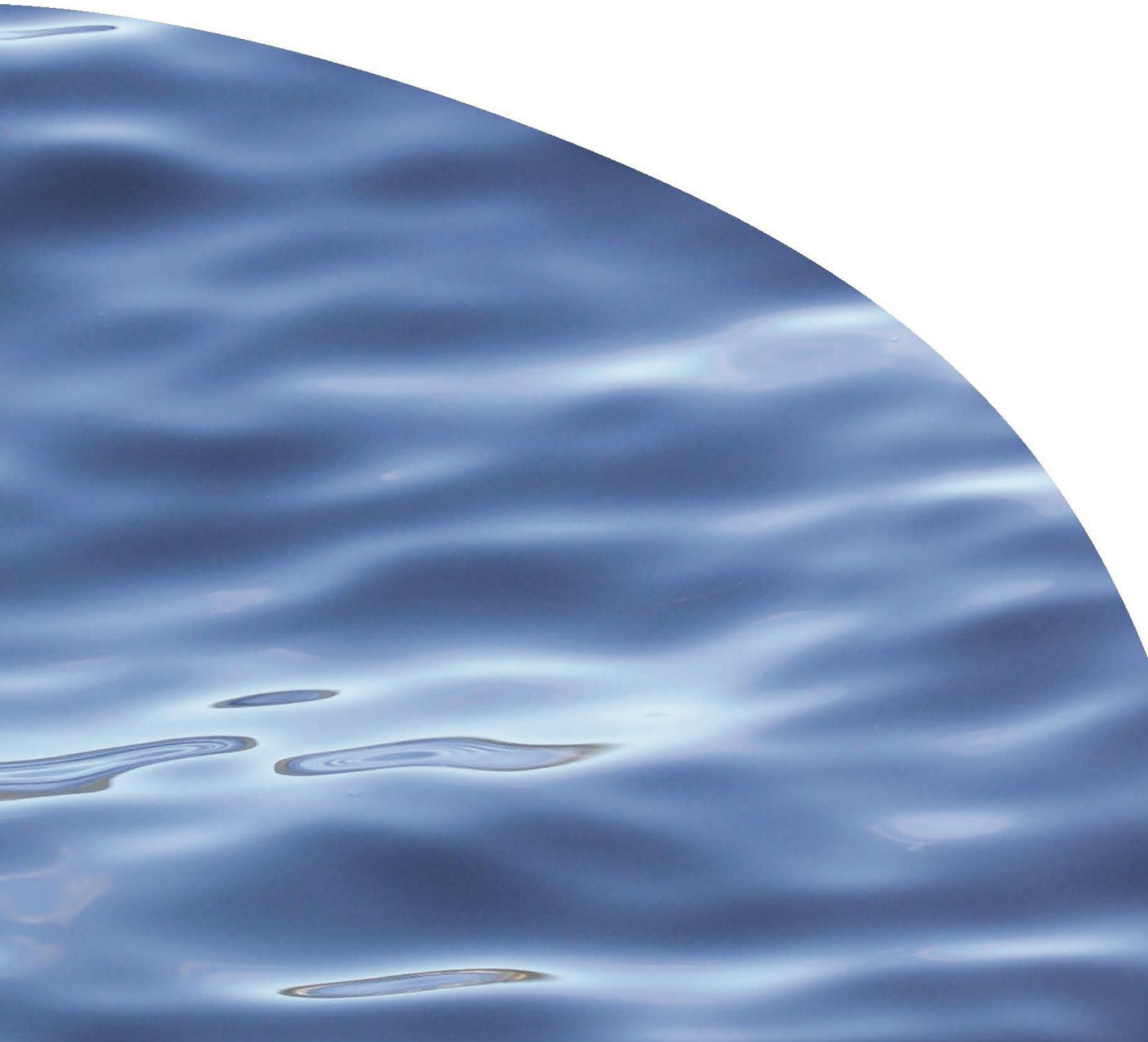

ASSESSMENT OF ANATOXIN LEVELS IN THE WATER OF RIVERS AFFECTED BY *PHORMIDIUM* BLOOMS

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Prepared for the Ministry for the Environment

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EXECUTIVE SUMMARY

Phormidium is a potentially toxic, mat-forming benthic cyanobacterium found in an increasing number of New Zealand rivers. In order to develop a National Objectives Framework attribute for benthic cyanobacteria (as part of the human health for recreation value in the National Policy Statement for Freshwater Management), the Ministry for the Environment has commissioned several studies to fill knowledge gaps. This has included a literature review to summarise the existing knowledge on benthic cyanobacteria and their associated toxins, an assessment of the environmental drivers of *Phormidium* blooms, and toxicology work to better understand the toxicity of the toxins (anatoxins) *Phormidium* produces. The anatoxins produced by *Phormidium* are largely contained within the cells, but previous research indicates that they can also be detected in overlying river water. In order to provide a more complete assessment of the human health risk associated with *Phormidium* in New Zealand and develop a National Objectives Framework attribute, more knowledge on the concentrations of anatoxins in river water is required.

The objective of this study was to assess anatoxin concentrations in rivers experiencing *Phormidium* blooms. Field studies were conducted in three rivers: Cardrona (Wanaka), Mataura (Southland) and Hutt (Wellington) in 2017. Every two to three hours over a 24- or 26-hour sampling period the following samples were collected: unfiltered water samples which were assessed for total anatoxins (i.e., toxins within cells and dissolved toxins) and the presence of *Phormidium* cells; filtered water samples which were analysed for dissolved toxins; and Solid Phase Absorbent Tracking Technology (SPATT) bags which were used to collect time-integrated toxin samples. Fifteen mat samples were also collected at each site.

Key findings were:

- Toxin concentrations in *Phormidium* mat samples from the Mataura and Hutt rivers were extremely high. The concentrations were over twice that recorded previously.
- Consistent with previous studies there was marked variability in toxin concentrations between mats over small distances.
- Anatoxins were detected in all total water and SPATT samples.
- In the Cardrona River no dissolved toxins were detected, suggesting that all toxins in the water column were contained within ‘free-floating’ *Phormidium* cells.
- In the Mataura and Hutt rivers, the majority of the toxins detected were dissolved.
- Temporal variability was observed in toxin concentrations in the river water and SPATT bags; however, there were no evident patterns between the sampling sites.

The result of this study demonstrates that when toxic *Phormidium* mats are present in a river there are also toxins in the water column. There appears to be a relationship between the concentrations in the mats and those detected in the water column,

although this requires further study. These data will provide valuable information for the development of human health risk assessment models related to *Phormidium* blooms in rivers.

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1. INTRODUCTION

Phormidium is a benthic cyanobacterium that grows as mats in certain rivers around New Zealand. *Phormidium* commonly produces toxins (anatoxins) which pose a human health risk through ingestion and skin contact. In order to develop a National Objectives Framework attribute for benthic cyanobacteria, the Ministry for the Environment (MfE) has commissioned several studies to fill knowledge gaps associated with *Phormidium* that are related to the human health for recreation value in the National Policy Statement for Freshwater Management (NPS-FM). This has included a literature review to summarise the existing knowledge on benthic cyanobacteria and their associated toxins (Wood et al. 2015a), an assessment of the environmental drivers of *Phormidium* blooms (Wood et al. 2017), and toxicology work to better understand the toxicity of the four main anatoxin congeners: anatoxin-a (ATX), dihydro-anatoxin-a (dhATX), homoanatoxin-a (HTX) and dihydro-homoanatoxin-a (dhHTX; Puddick et al. 2017a, 2017b). The anticipated final output of the toxicity project will be advice on an assessment of the human health risk associated with *Phormidium* blooms in New Zealand rivers. However, in order to conduct this risk assessment, more knowledge on the concentrations of anatoxins in bloom-affected river water is required.

Wood et al. (2011) evaluated the potential of an *in situ* method known as solid phase adsorption toxin tracking (SPATT) for collecting and concentrating anatoxins in river water. In addition to laboratory validation they undertook a three-day field study in the Waipoua River (Wairarapa) during a *Phormidium* bloom. During this study, anatoxins were detected in all SPATT samples and in one of five river water grab samples. However, samples were only collected at five time points over the sampling period, limiting knowledge on the temporal variability in toxin concentrations. As the study was only undertaken at one site it is unknown whether the results are representative of other rivers in New Zealand.

The objective of the present study (as commissioned by MfE) was to assess anatoxin concentrations in rivers experiencing *Phormidium* blooms. The study involved the collection of water samples (total toxins; i.e., including toxins present inside *Phormidium* cells in the water column and dissolved toxins), filtered water samples (dissolved toxins), the use of SPATT bags to collect time-integrated samples, and an assessment of toxin concentrations in *Phormidium* mats from three rivers. Samples were collected over a 24- or 26-hour period. The rationale for this timing was that previous research has shown large variations in pH (7.8-9.7) and dissolved oxygen (0.05 to 17.2 mg L^{-1}) in *Phormidium* mats over diurnal cycles due to photosynthesis (daytime) and respiration (night time; Wood et al. 2015b). We theorised that factors associated with these processes may cause the lysis of *Phormidium* cells, and result in toxins been released in pulses at certain times of the diurnal cycle.

2. METHODS

2.1. Sampling sites

The field studies were conducted at three sites:

1. Cardrona River (Wanaka). This site was directly upstream of the State Highway bridge on the Wanaka-Luggate Highway 6 about 3 km east of Wanaka (44°41'57"S, 169°10'38"E). The SPATT bags (see below for description of these) were deployed in a pool (0.5 m deep × 5 m wide; Figure 1). For 40 m upstream from the SPATT bag deployment site, the stream was 5-8 m wide, and largely in riffle habitat with an average depth of 0.4 m. The substrate was small to medium cobbles. About 200 m upstream of the SPATT site the stream emerged from the gravel bed (i.e., there was no water flowing above-ground upstream; Figure 1).

2. Mataura River (Southland). This site was approximately 200 m upstream of the road bridge on Mataura Island Road, about 30 km east of Invercargill (46°23'25"S, 168°46'35"E). The stream was approximately 70 m wide and in run habitat. The SPATT bags were deployed 10 m from the true left bank (depth 0.5 m). The substrate was small cobbles and gravel (Figure 2).

3. Hutt River (Wellington). This site was approximately 800 m upstream of the Silverstream Road bridge, about 10 km north east of Lower Hutt (41° 8'24"S, 175° 0'17"E). The stream was approximately 50 m wide and in run habitat. The SPATT bags were deployed 20 m from the true right bank (depth 0.6 m). The substrate was small cobbles (Figure 3).

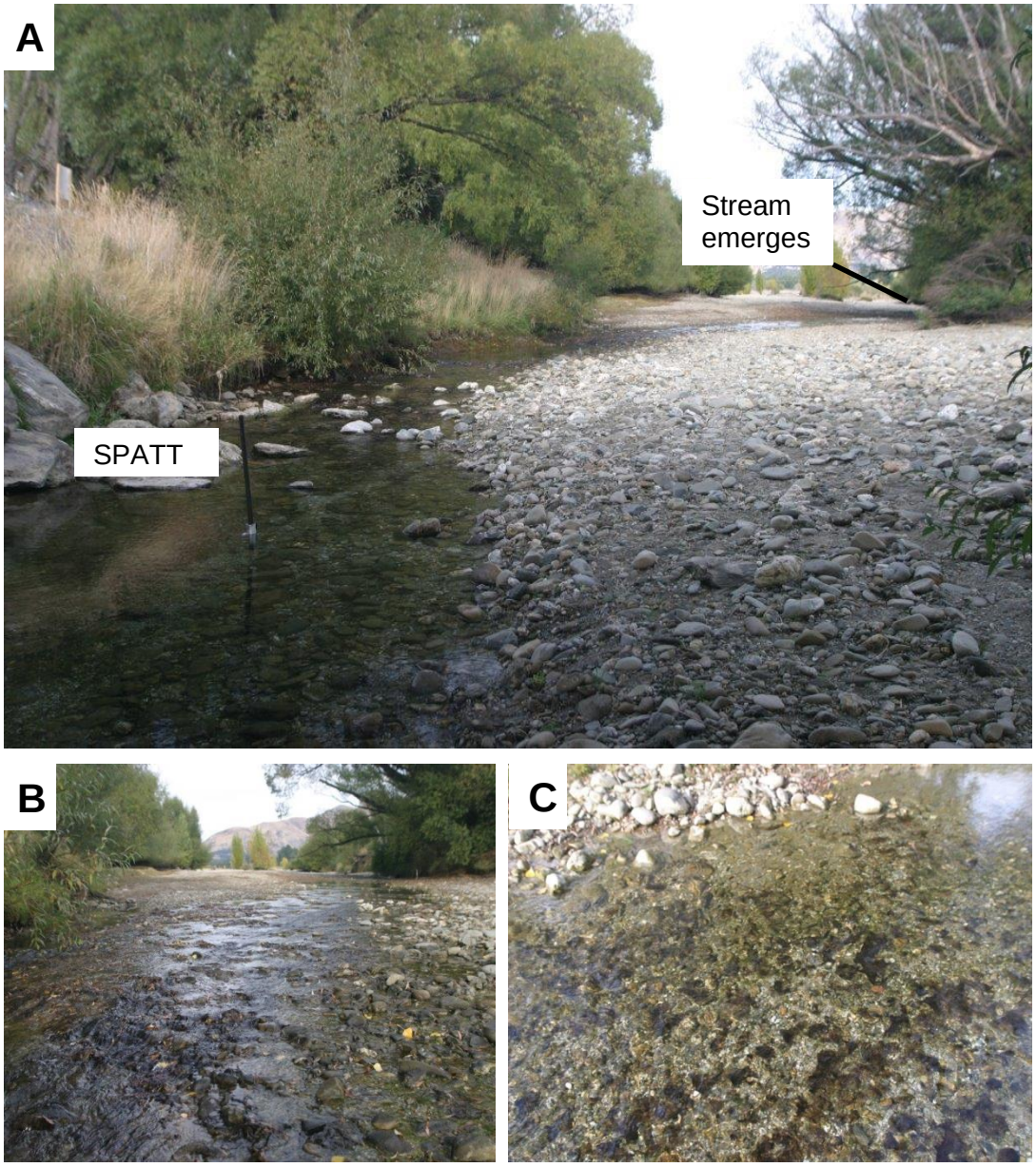

Figure 1. Sampling site at the Cardrona River. (A) Overview of site with steel stake and SPATT bags in foreground. (B) and (C) *Phormidium* mats in shallow riffle, approximately 20 m and 40 m upstream from SPATT deployment site, respectively.

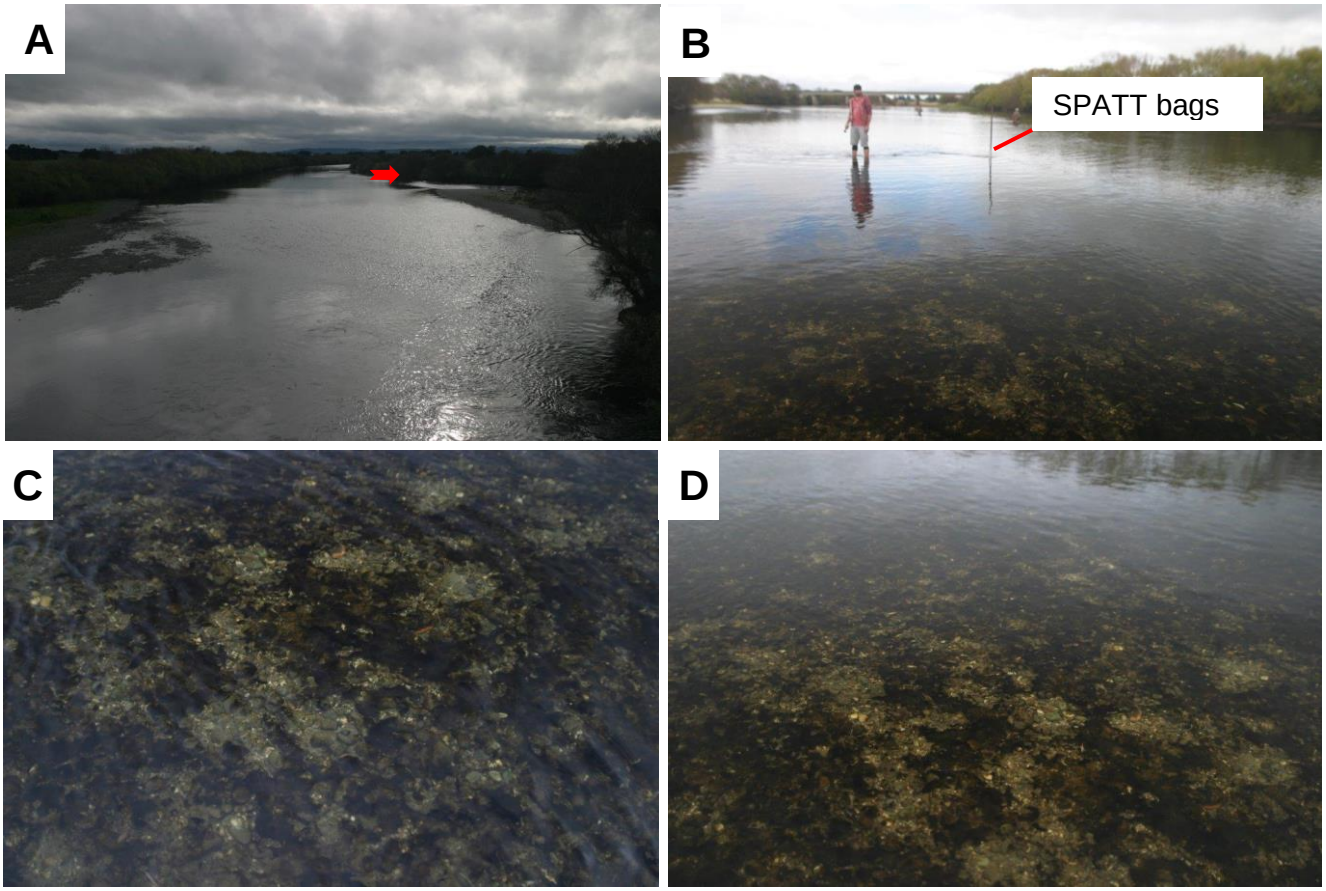

Figure 2. Sampling site at the Mataura River. (A) Looking upstream from the Mataura Island Road bridge the sampling site is approximately 200 m upstream (marked with arrow), (B) SPATT bag deployment site, note the Mataura Island Road bridge in the background, and (C) and (D) *Phormidium* mats on the river bed (depth c. 0.5 m).

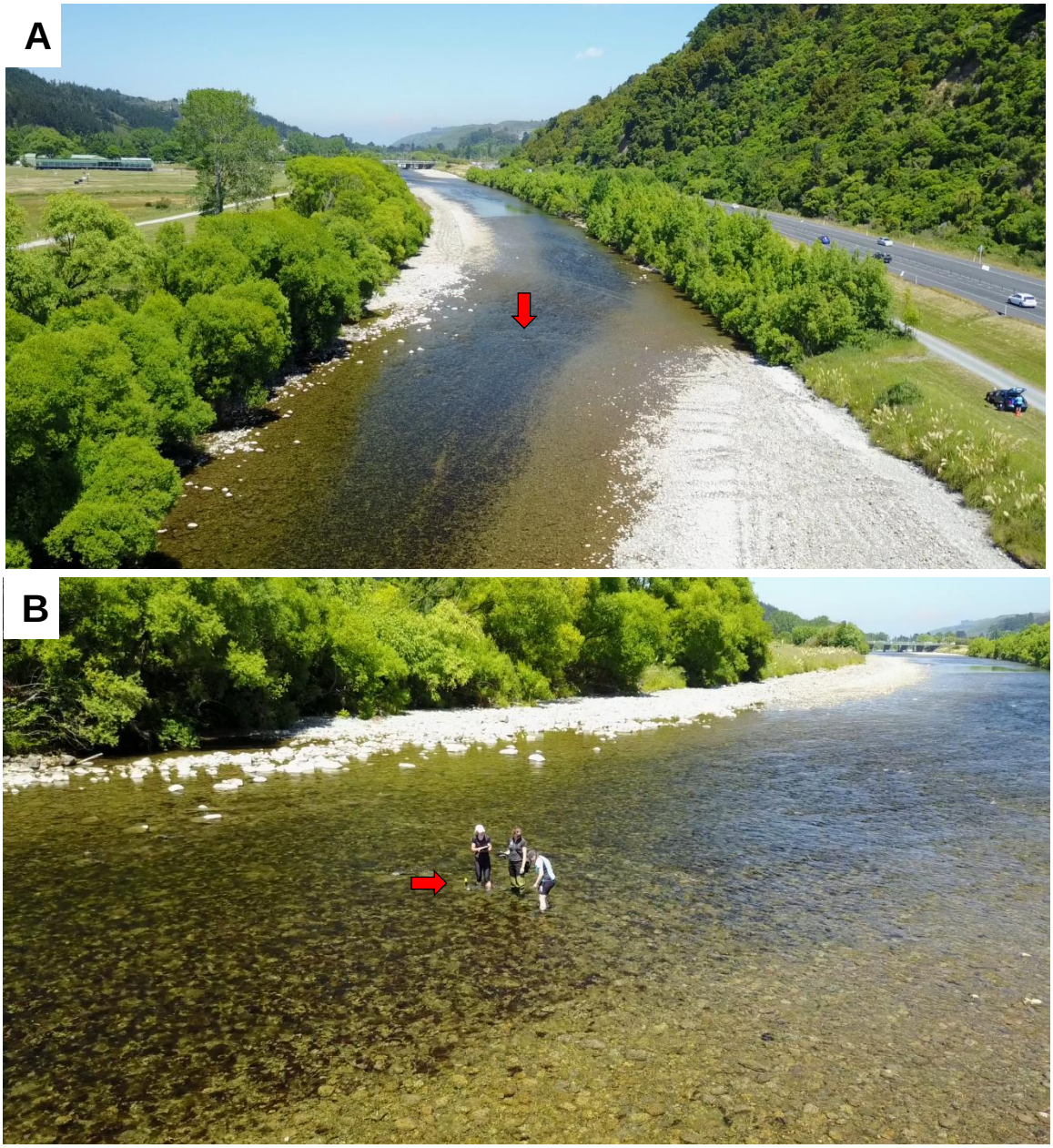

Figure 3. Sampling site at the Hutt River. (A) Aerial photo looking downstream with the Silverstream Road bridge in background (approximately 800 m), (B) study site in the Hutt River. Red arrows mark SPATT deployment site. Photos: Greater Wellington Regional Council.

2.2. Field sampling

2.2.1. Site characterisation and Phormidium *mat sampling*

The method used to estimate *Phormidium* differed between the rivers due to their geomorphology and depth. The Cardrona River was too shallow to use a bathyscope and the Mataura was too deep. The depth of the Mataura also prevent transects spanning the entire width of the river.

Cardrona River — at the end of the 24-hr sampling period, *Phormidium* cover was visually assessed by estimated the cover within a 0.5 m-wide strip along five transects perpendicular to the bank and spanning the width of the stream. These were situated 10, 22, 26, 29 and 38 m upstream of the SPATT deployment site. Three *Phormidium* samples were collected at approximately equal spacing along each transect by scraping a mat from one rock into a sterile tube (2 mL). These were frozen (−20°C) until extracted for toxin analyses.

Mataura River — two transects were positioned 2 and 7 m downstream of the SPATT site and perpendicular to the bank. The transects extended 31 and 24 m out into the river, beyond which the river depth made it unsafe to make further measurements. *Phormidium* cover was visually estimated (within a 0.5 m^2 area) every 3 m and samples were collected by lifting a rock out of the water and scraping the mat into a sterile tube (2 mL). This was undertaken at 3 m intervals for the first 21 m on Transect One, and the first 24 m on Transect Two. These were frozen (−20°C) until extracted for toxin analyses.

Hutt River — at the end of the 24-hr sampling period, *Phormidium* cover was visually assessed along three transects positioned 10, 20 and 30 m upstream of the SPATT site and perpendicular to the bank. *Phormidium* cover was visually estimated at five equidistant points to a distance of 50 m using a bathyscope (Model 0800, Nuova Rade, Italy). Samples were collected at each measurement point by lifting a rock out of the water and scraping the mat into a sterile tube (2 mL). These were frozen (−20°C) until extracted for toxin analyses.

2.2.2. River water samples

Triplicate water samples (c. 15 mL) were collected from the surface adjacent and 1 m either side of the SPATT bags at each sampling time point (Table 1). Three sub-samples were taken: (1) 1 mL of water was frozen (−20°C) for total anatoxin analysis; (2) 1 mL was syringe-filtered (0.45 µm) directly into a liquid chromatography (LC) vial and the filtrate was frozen (−20°C) for extracellular anatoxin analysis; and (3) c. 10 mL of water was preserved using Lugol's iodine for microscopic analysis. A second water sample (c. 50 mL) was collected adjacent to the SPATT bags and syringe-filtered (Whatman GF/C, c. 1.6 µm pore size) and the filtrate frozen (−20°C) for dissolved nutrients analysis.

Table 1. Time and date of SPATT bag deployment and subsequent sampling each river.

	Cardrona River			Mataura River			Hutt River		
Sample	**Date**	**Time**	**Elapsed Time hrs**	**Date**	**Time**	**Elapsed Time hrs**	**Date**	**Time**	**Elapsed Time hrs**
Start	10 Apr 17	1310	0	12 Apr 17	1045	0	28 Nov 17	1000	0
1	10 Apr 17	1510	2	12 Apr 17	1245	2	28 Nov 17	1200	2
2	10 Apr 17	1710	4	12 Apr 17	1445	4	28 Nov 17	1400	4
3	10 Apr 17	1910	6	12 Apr 17	1645	6	28 Nov 17	1630	6.5
4	10 Apr 17	2110	8	12 Apr 17	1845	8	28 Nov 17	1830	8.5
5	10 Apr 17	2310	10	12 Apr 17	2045	10	28 Nov 17	2030	10.5
6	11 Apr 17	0110	12	12 Apr 17	2245	12	28 Nov 17	2200	12
7	11 Apr 17	0310	14	12 Apr 17	1245	14	29 Nov 17	0000	14
8	11 Apr 17	0510	16	13 Apr 17	0345	17	29 Nov 17	0200	16
9	11 Apr 17	0710	18	13 Apr 17	0645	20	29 Nov 17	0400	18
10	11 Apr 17	0910	20	13 Apr 17	0845	22	29 Nov 17	0600	20
11	11 Apr 17	1110	22	13 Apr 17	1045	24	29 Nov 17	0800	22
12	11 Apr 17	1310	24	13 Apr 17	1245	26	29 Nov 17	1000	24

2.2.3. SPATT bags design and deployment

SPATT bags were constructed from two 7 × 7 cm square pieces of 5 µm polyester mesh with one gram of Strata-X (Phenomenex, Auckland, New Zealand) placed in each bag. The bags were attached with a hose clip to a holding tube made from aluminium alloy (Figure 4) as described in Wood et al. (2011). Three SPATT holding tubes were attached to one side of a larger aluminium alloy mounting tube using 4 mm diameter screws (Figure 4). The mounting tube was then placed over a steel stake (waratah) embedded into the river bed and secured in position with a clamping screw. The top holding tubes were positioned approximately 10 mm below the river surface.

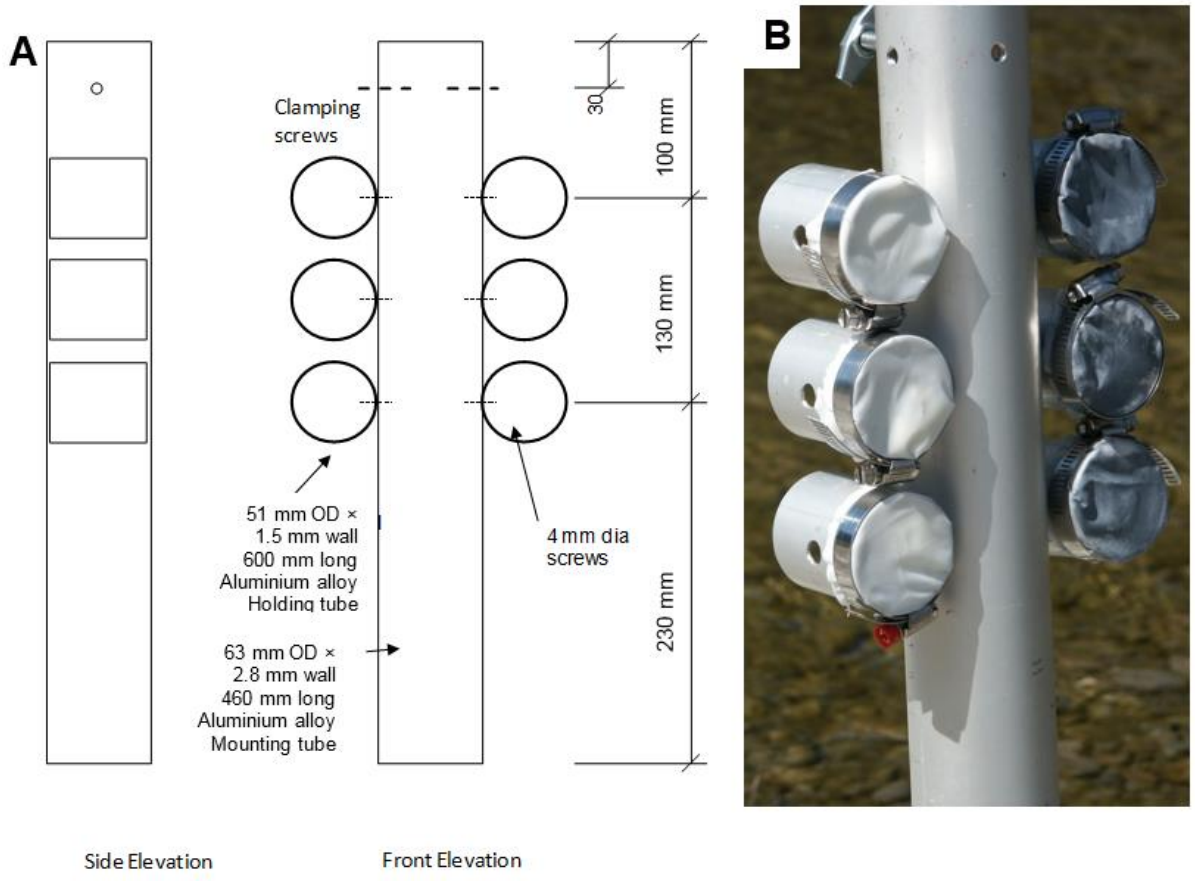

Figure 4. (A) Schematic diagram showing SPATT bag holding tubes and mounting system. (B) SPATT bags on mounting tube. OD = outside diameter. Note: That the SPATT bags on the right side of the image in B contain powdered activated carbon not Strata-X and during the present study SPATT bags were only deployed on one side of the aluminium alloy mounting tube.

Immediately prior to deployment, each SPATT bag was soaked in 70% ethanol for at least 1 hr. A single mounting tube with three SPATT bags attached was deployed and at each sampling point (Table 1) the SPATT bags were removed from the river and replaced with new ones. The SPATT bags were then frozen (−20°C) in 20 mL glass vials for later extraction and toxin analysis. Six controls were included in the project, three that were soaked in fresh ethanol in the laboratory, and three that were soaked in the ethanol used throughout the field studies.

2.2.4. Physicochemical measurements

In the Cardrona and Mataura rivers, temperature was measured at 5 min intervals using a temperature logger (HOBO, Onset) positioned directly below the SPATT bags. The loggers were not available to deploy in the Hutt River. Dissolved oxygen, conductivity, pH and spot temperatures were measured at all sites using a handheld water quality sonde (YSI ProPlus, YSI Inc., OH, USA) adjacent to the SPATT

deployment sites at each sampling point (Table 1). The water velocity at the midpoint of the SPATT bags was measured using a hand held velocity meter (Marsh-McBirney, HACH, CO, USA) at the start, middle and end of the sampling periods in the Cardrona and Mataura, and the start and end in the Hutt River.

2.3. Laboratory analysis

2.3.1. Extraction of total anatoxins from water

The water samples collected for total anatoxin analysis (2 mL) were supplemented with concentrated formic acid (2 µL) and placed in a sonicating bath for 30 min. The samples were then re-frozen (−20°C) and sonicated again (whilst thawing). The extracts were clarified by centrifugation (17,000 ×g, 5 min), transferred to LC vials and stored at −20°C until analysis.

2.3.2. Phormidium *mat anatoxin extractions*

Frozen *Phormidium* mat samples were defrosted, transferred to 20 mL glass vials re-frozen at −70°C and lyophilised (Gamma 1-16 LSC freeze-drier; Martin Christ Gefriertrocknungsanlagen, Germany). Lyophilised material was ground with a sterile metal spatula before an aliquot (c. 10 mg) was weighed into a micro-centrifuge tube and suspended in 1 mL of 0.1% formic acid (made up in Milli-Q water). These samples were mixed on a vortex mixer for 30 s, frozen at −20°C and thawed in a sonicator bath for 30 min. This freeze-thaw process was repeated two more times before the samples were clarified by centrifugation (12,000 ×g, 5 min). The clarified extracts were diluted 1/20 in 0.1% formic acid and stored in LC vials at −20°C until anatoxin analysis.

2.3.3. SPATT bag anatoxin extractions

The SPATT samples and SPATT controls were brought to ambient temperature and 10 mL of methanol (MeOH) containing 0.1% formic acid was added. The samples were gently rocked in the dark for 2 h at 4°C with the polyester mesh and Strata-X material completely submerged. After the polyester mesh was removed, the Strata-X material was allowed to sediment on the bottom of the vial. An aliquot (5 mL) of the supernatant was transferred to a new 20 mL glass vial and dried under a stream of nitrogen gas with heating at 40°C. The concentrated extract was resuspended in 1 mL of Milli-Q water containing 0.1% formic acid and centrifuged (10,000 ×g, 1 min). The clarified extract was transferred to a LC vial avoiding any residual Strata-X material and stored at −20°C until anatoxin analysis. Because two of the SPATT deployment times in the Mataura River were for 3 hrs, and two in the Hutt River were 2.5 and 1.5 hrs, as opposed to 2 hrs (Table 1), the data were normalised to the amount of anatoxin accumulated in each SPATT bag per hour.

2.3.4. Anatoxin analysis

Samples were analysed for dhATX, dhHTX, HTX and ATX, using high-performance liquid chromatography-mass spectrometry (HPLC-MS) as described in Wood et al. (2016). Calibration was via an external standard curve constructed using dilutions of ATX certified reference material (National Research Council, Canada; 0.5–20 ng mL^{-1} in 0.1% formic acid). A relative response factor of 1, using ATX as the calibration reference was used to quantify HTX, dhATX and dhATX.

2.3.5. Morphological examination of water samples for Phormidium filaments

Aliquots of the Lugol's preserved water samples (10 mL) were settled (> 6 hrs) in Utermöhl chambers (Utermöhl 1958). Each sample was scanned for the presence of *Phormidium* filaments using an inverted Olympus microscope at 400× magnification (IMT-2, Olympus, Wellington, New Zealand). When *Phormidium* filaments were observed their length was measured using CellSens (Version 1.0, Olympus). The number of cells present in each sample was then estimated based on published cell length data (Harland et al. 2014).

2.3.6. Nutrient analysis

Samples were analysed at Hill Laboratories (Hamilton, New Zealand) with a Lachat Quickchem® flow injection analyser (FIA+8000 Series, Zellweger Analytics, Inc.) using APHA (2012) 4500 methods for nitrite (NO_2-N), nitrate (NO_3-N), ammonium (NH_4-N) and dissolved reactive phosphorus (DRP). The limits of quantification were 0.002 mg L^{-1} for NO_2-N, 0.002 mg L^{-1} for NO_3-N, 0.01 mg L^{-1} for NH_4-N, and 0.004 mg L^{-1} for DRP.

2.3.7. Statistical analysis

Statistical analyses were performed using the R statistical package (http://www.R-project.org/). Normality was checked through inspection of Quantile-Quantile plots and conducting a Shapiro-Wilk test. As normality was not met for the dissolved, total and SPATT anatoxin concentrations, a log transformation was undertaken. Statistically significant differences between sampling points for the water and SPATT bag anatoxins results were assessed at each site separately using a one-way analysis of variance (ANOVA). A Tukey honest significant difference pairwise post-hoc test was used to determine differences between pairs of samples if a significant result was revealed.

3. RESULTS

3.1. *Phormidium* cover and toxins in *Phormidium* mat samples

In the Cardrona River the average *Phormidium* cover in the 38 m upstream of the SPATT deployment sites was 23% (range 10–30%). In the Mataura River the average *Phormidium* cover along the two transects was 51% (range 25–80%). In the Hutt River average *Phormidium* cover along the three transects was 42% (range 2–90%).

Anatoxins were detected in all *Phormidium* mat samples, although the concentrations and proportions of each anatoxin congener varied within and between the three study sites (Figure 5). The average summed anatoxin concentration (i.e., the concentration of all four congeners combined) in the Cardrona River samples was 40 mg kg^{-1} dry weight (dw; range 2 to 239 mg kg^{-1} dw), compared to 515 mg kg^{-1} dw (range 10 to 1684 mg kg^{-1} dw) for the Mataura River samples and 555 mg kg^{-1} dw (range 12 to 2116 mg kg^{-1} dw) for the Hutt River (Figure 5). Dihydro-anatoxin-a was the dominant congener in all mat samples from the Cardrona River and in all but two mats in the Hutt River, (Figure 5B). In contrast, with the exception of three mats, dhHTX was the most abundant congener in the mat samples in the mat samples from the Mataura River (Figure 5). The Hutt River mats contained relatively high proportions of HTX (range 17% to 49%; Figure 5). Full toxin results, including the concentration of each congener are provided in Appendix 1.

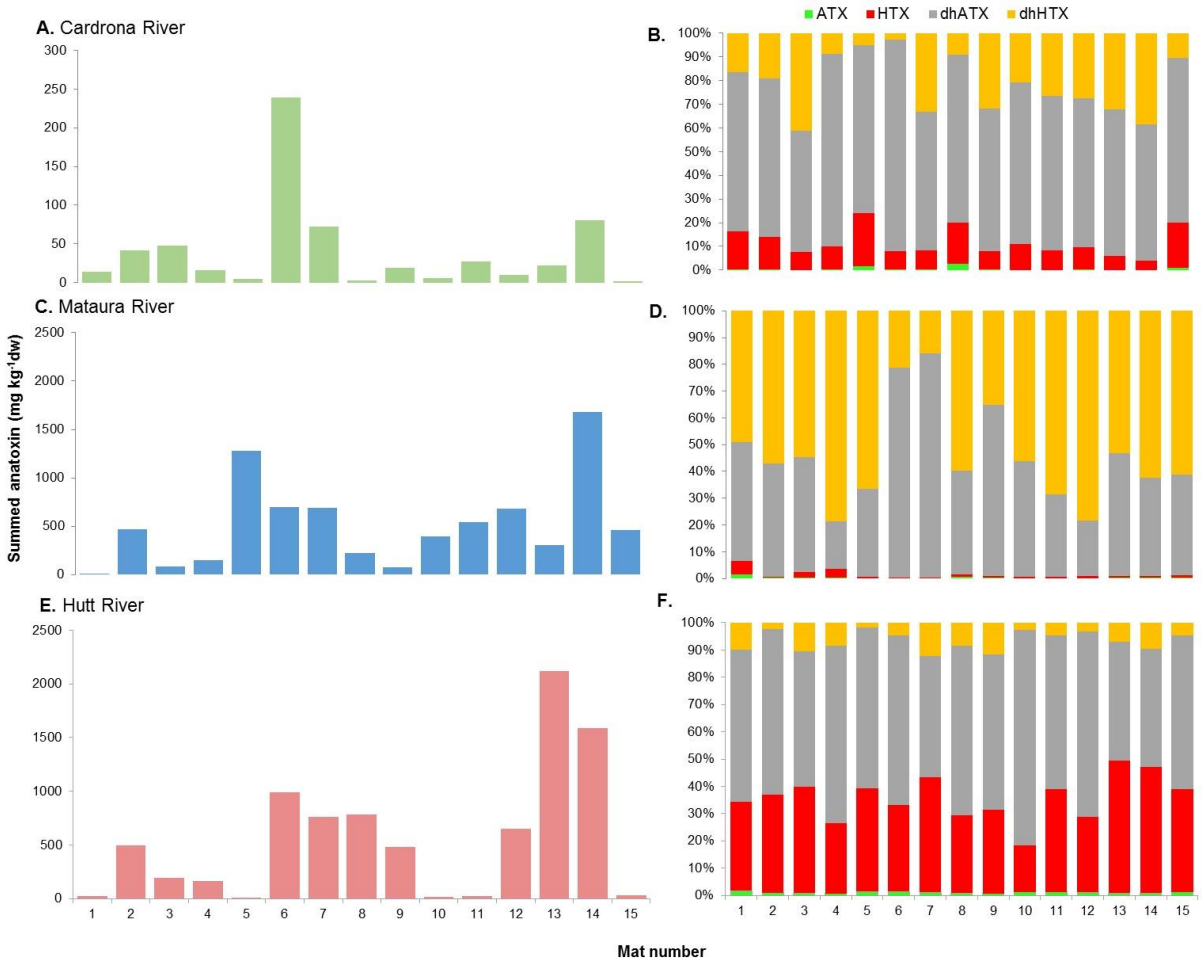

Figure 5. Summed anatoxin concentrations (i.e., the total of the four congeners; anatoxin-a (ATX), homoanatoxin-a (HTX), dihydro-anatoxin-a (dhATX), and dihydro-homoanatoxin-a (dhHTX)) in 15 *Phormidium* mat samples collected from the Cardrona River on 11 April 2017, (A and B), the Mataura River on 12 April 2017 (C and D) and the Hutt River on 29 November 2017 (E and F). Panels B, D and F show the percentage each congener in the mat samples. Note the difference in the y-axis scales for A, C and E.

3.2. Total and dissolved anatoxins in water samples

In the total anatoxin (the sum of intracellular and dissolved anatoxin) samples from the Cardrona River, only low concentrations of dhATX (≤ 0.12 ng mL^{-1}) were detected (Figure 6A). One-way ANOVA analysis showed there was no significant difference in the total anatoxin concentrations over the sampling period ($P = 0.053$). No anatoxins were detected (limit of detection = 0.03 ng mL^{-1}) in the dissolved anatoxin samples collected from the Cardrona River.

On average, total anatoxin concentrations were nine-fold lower in the Cardrona River water samples those from the Mataura River (Figure 6), with a maximum value of 0.91 ng mL^{-1} recorded (1045 h, 13 April 2017). One-way ANOVA analysis showed there was a significant difference among the Mataura River water samples over the sampling period ($P < 0.001$). The Tukey HSD post-hoc test demonstrated that this was largely due to the 1045 h sample (12 April) being significantly lower than all others, and the 1245 h samples (12 and 13 April 2017) and the 1045 h sample on 13 April 2017 being significantly higher than all other samples (Appendix 2). In contrast to the Cardrona water samples, the majority of the toxins detected in the Mataura River samples were in the dissolved form, not intracellular (Figure 6C). The most dominant anatoxin congener in the samples was dhATX, with the other congeners being detected in approximately equal proportions (Figure 6D).

In general the total anatoxin concentrations in the Hutt River water samples (Figure 6E) were slightly lower than those in the Mataura River (Figure 6B), with a maximum value of 0.22 ng mL^{-1} recorded (1000 h, 28 November April 2017). One-way ANOVA analysis showed there was a significant difference among the water samples over the sampling period ($P < 0.001$). The Tukey HSD post-hoc test demonstrated that this was largely due to the 2200 h sample (28 November) being significantly higher than the early afternoon (28 November) and early morning samples on 29 November, and the 0600, 0800 and 1000 h samples (29 November 2017) being significantly lower than the 1430 and 1630 samples (28 November; Appendix 2). With the exception of the 1000 sample (28 November), where 0-54% of the toxins were intracellular, the remainder were in the dissolved form (Figure 7F). The most dominant anatoxin congener in the samples was dhATX, followed by HTX (Figure 7G).

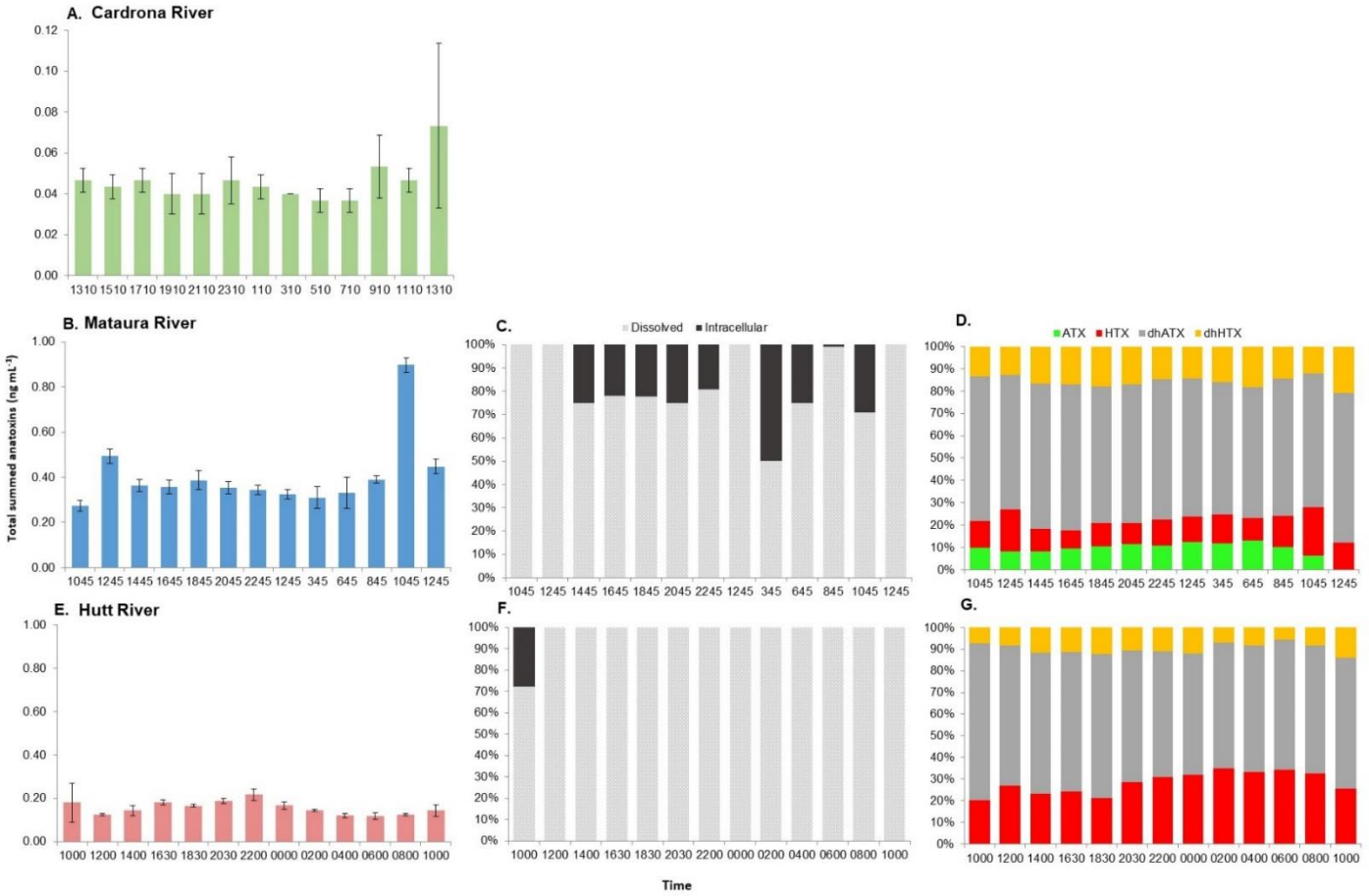

Figure 6. Average concentration of summed anatoxin concentrations (i.e., the total of the four congeners; anatoxin-a (ATX), homoanatoxin-a (HTX), dihydro-anatoxin-a (dhATX), and dihydro-homoanatoxin-a (dhHTX)) in the total anatoxin (i.e., intracellular and dissolved) water samples from the (A) Cardrona River (10 and 11 April 2017), (B) Mataura River (12 and 13 April 2017) and (E) the Hutt River (28 and 29 November 2017), the proportion of summed anatoxin that were dissolved and intracellular in the (C) Mataura and (F) Hutt, and the percentage of each congener in the samples from the (D) Mataura River and (G) Hutt River. Only one graph is show for Cardrona as no dissolved toxins were detected and the only congener detected in the total samples was dhHTX. Error bars show ± one standard deviation (n = 3).

3.3. Morphological analysis of *Phormidium* filaments in the water samples

Phormidium cells were identified in three, eight and 31 of the 39 water samples collected from the Cardrona River, Mataura River and Hutt River, respectively. The estimated cell concentrations ranged from 0 to 38 cells mL^{-1} for the Cardrona River, 0 to 33 cells mL^{-1} for the Mataura River, and 0 to 34 cells mL^{-1} for the Hutt River (Appendix 3).

3.4. Anatoxins in SPATT bags

No anatoxins were detected in the laboratory controls (data not shown). Low concentrations (< 1.6 ng of summed anatoxins per g of Strata-X^{-1} hr^{-1}) of anatoxins were detected in the field ethanol extraction controls that were undertaken at the end of the field experiments, and these values were removed from the Mataura River SPATT data.

All of the SPATT bags samples collected from the three study sites contained anatoxins (Figure 7). On average, anatoxin concentrations in the SPATT bags from the Mataura and Hutt rivers were 20-fold higher than those deployed in the Cardrona River (Figure 7A, C, E).

Anatoxin concentrations in the SPATT bags from the Cardrona River varied significantly over the 24-hr sampling period (one way ANOVA; $P < 0.001$) (Figure 7A). Tukey HSD post-hoc tests showed that the SPATT bags collected at 0710 h contained significantly higher anatoxin concentrations than all other sampling times, and those at 0910 h and 1110 h were significantly higher than all others except the 0510 h bags (Appendix 4). In general, dhATX was the most abundant congener detected, with the exception of three samples where ATX dominated (Figure 7B). Anatoxin concentrations in the SPATT bags in the Mataura River also varied significantly over the 26-hr sampling period, although the differences were not as pronounced (one way ANOVA; $P < 0.01$) (Figure 7C). Tukey HSD post-hoc tests showed that all of the difference could be attributed to the SPATT bags collected at 1045 h, which contained significantly higher levels of anatoxin than those collected between 1845 and 0345 h (Appendix 4). The most abundant congener in the SPATT samples from the Mataura River site was dhATX (Figure 7D).

Anatoxin concentrations in the SPATT bags in the Hutt River varied significantly over the 24-hr sampling period (one way ANOVA; $P < 0.001$) (Figure 7E). Tukey HSD post-hoc tests showed that all of the difference could largely be attributed to the SPATT Samples be attributed to the SPATT bags collected at 1200, 1400 and 1630 h, which contained significantly lower levels of anatoxin than most other samples (Appendix 4). The most abundant congener in all samples was dhHTX (Figure 7F).

In general, at each site the highest anatoxins concentrations recorded in the water samples also aligned with the highest values measured in the SPATT samples. For example, the 1045 h samples in the Mataura, and 2030 h to 2200 h samples in the Hutt River (Figure 6 and Figure 7). However, there was no consistent temporal pattern in fluctuations or peaks of toxins in either the water or SPATT samples among sites (Figure 6 and 7).

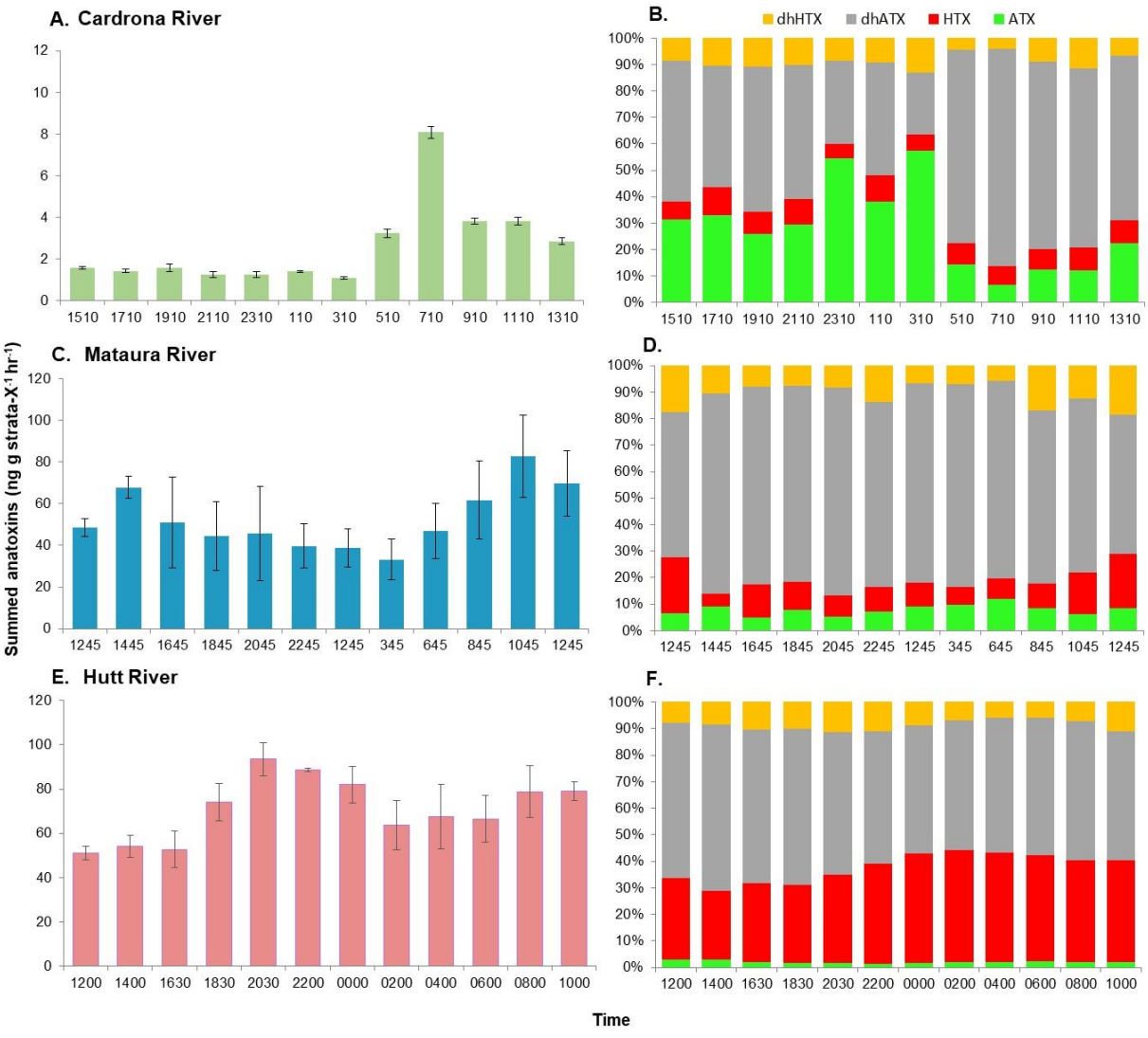

Figure 7. Average concentration of summed anatoxins (i.e., the total of the four congeners; anatoxin-a (ATX), homoanatoxin-a (HTX), dihydro-anatoxin-a (dhATX), and dihydro-homoanatoxin-a (dhHTX)) from SPATT bags deployed in the (A and B) Cardrona River 10-11 April 2017, (C and D) Mataura River 12-13 April 2017 and (E and F) Hutt River 28-29 November 2017. Error bars show ± one standard deviation (n = 3). Note difference in x- and y-axis scales. (B) (D) and (F) show the percentage of each anatoxin congener in the SPATT bag samples.

3.5. Physiochemical characterisation of sites

Over the sampling period water temperature in the Cardrona River varied between 12.03–13.85°C (logger data), 11.14–12.59°C in the Mataura River (logger data) and 17.9–22.6 °C in the Hutt River (spot measurements; Appendix 5). Dissolved oxygen (median 73% at Cardrona [range 71.1-82%], 101% at Mataura [89.1-122], 91.8% at Hutt [86-112], conductivity (median 108.4 µS cm^{-1} at Cardrona [range 108.3-108.7 µS cm^{-1}], 119.3 µS cm^{-1} at Mataura [range 113.8-132 µS cm^{-1}], 106.5 µS cm^{-1} at Hutt [range 104.5-110.4 µS cm^{-1}] and pH (median 7.2 at Cardrona [range 7-7.6], 7.4 at Mataura [range 7.2-8.3], not measured at Hutt) remained relatively constant across the sampling period (Appendix 6).

The average water velocity at the position of the SPATT bags was 0.07 $m^3 s^{-1}$ in the Cardrona River (range 0.06-0.08 $m^3 s^{-1}$), 0.1 $m^3 s^{-1}$ in the Mataura River (range 0.09-0.11 $m^3 s^{-1}$), and 0.58 $m^3 s^{-1}$(range 0.57-0.59 $m^3 s^{-1}$), in the Hutt River.

The median (n = 13) dissolved inorganic nitrogen (sum of NO_2-N, NO_3-N and NH_4-N) concentrations in the Cardrona, Mataura and Hutt rivers were 0.355 mg L^{-1} (range 0.136 to 0.464 mg L^{-1}), 0.572 mg L^{-1} (range 0.100 to 1.114 mg L^{-1}), and 0.143 mg L^{-1} (range 0.042 to 0.244 mg L^{-1}) respectively. Median DRP concentrations in the Mataura River were 0.007 mg L^{-1} (range 0.005 to 0.011 mg L^{-1}). In the Cardrona River only one sample was above the limit of detection with a value of 0.004 mg L^{-1}, and in the Hutt River all samples were below the limit of detection (< 0.004 mg L^{-1}).

4. DISCUSSION AND CONCLUSIONS

4.1. Anatoxin concentrations in mats

In all three rivers there was marked variability in toxin concentrations between mats over small distances; e.g., 120-fold differences were observed at the Cardrona River within a c. 120 m^2 area, 168-fold at the Mataura River along two c. 20 m transects, and 176-fold along three c. 50 m transects at the Hutt River. This result is in congruence with previous research (Heath et al. 2010; Wood et al. 2010, 2012).

Three theories have been proposed to explain the variability of anatoxin concentrations observed in *Phormidium* mats:

1. The relative abundance of co-occurring organisms and/or inorganic material varies in the mats. Although mats are dominated by *Phormidium*, they may also contain many other organisms (e.g., bacteria and eukaryotic algae), and inorganic matter, (e.g., sediment), bound together by extracellular polymeric substances (Hart et al. 2013; Brasell et al 2015; Wood et al. 2015b). The quantity and composition of these may vary over time and between sites.
2. Conditions within the mat (e.g., dissolved oxygen, pH) or surrounding water (e.g., nutrient concentrations) cause an up- or down-regulation in the amount of anatoxins produced per cell.
3. Toxic and non-toxic *Phormidium* genotypes co-occur in the mats, and their relative abundance and amount of toxin they produce affects total anatoxin concentrations (Wood et al. 2012).

In a recently published study, Wood and Puddick (2017) provide strong evidence to demonstrate that the variability in anatoxin concentrations among mats is primarily due to the abundance of toxic genotypes. In their study, they selected three mats in the Cardrona and Mataura rivers during the sampling period of this study and sampled these repeatedly in parallel with water and SPATT samples. A method was employed which combined LC-MS analysis of anatoxin concentrations and digital droplet polymerase chain reaction (PCR) with *Phormidium*-specific *anaC* primers (a gene involved in anatoxin synthesis) to measure absolute quantities of toxin gene copies. This allowed for the determination of anatoxin quotas (toxins per cell) in field samples. The data were compared to the conventionally-used anatoxin concentrations (assessed per dry weight of mat). Anatoxin concentrations in the mats varied 59-fold and 303-fold in the two rivers. However, when converted to anatoxin quotas there was markedly less variability (42- and 16-fold). Although Wood and Puddick (2017) do not rule out the contribution of other factors to the variability in anatoxin concentration, they suggest the much lower variability in toxin quotas clearly shows one of the key contributors to differences in the anatoxin concentrations of mat samples is the abundance of toxic genotypes in the *Phormidium* mats.

Toxins concentrations in *Phormidium* mat samples from the Mataura and Hutt rivers were extremely high. The highest values measured were over twice that recorded previously in a nationwide collation of data (previous maximum = Oreti River, Southland on 1 October 2010, 771 mg kg^{-1} dw, McAllister et al. 2016). Comparisons of these values with international data is challenging as most studies have used different toxin detection methods (i.e., ELISA) or data is from cultured material (e.g., Fetscher et al. 2015; Cantoral Uriza et al. 2017). Despite these caveats and based on our literature searches these values appear to be much higher than reported in other countries. In general the most abundant congeners in the mat samples were dhATX and dhHTX (Figure 5). This is consistent with other studies in New Zealand, although previous research has occasionally detected HTX in high abundance (McAllister et al. 2016), which was observed in some of the Hutt River samples during the present study.

4.2. Anatoxins in water and SPATT samples

Anatoxins were detected in all water (total toxins) and SPATT samples. This is in contrast to Wood et al. (2011) who detected toxins in only one of five grab samples from the Waipoua River (Wairarapa). Toxin concentrations in the mats in the Mataura and Hutt rivers were generally much higher than those in Wood et al. (2011), but the levels in Cardrona were similar; therefore, it is unlikely that toxins concentrations is the main reason for the differences. A possible reason for this could be an increase in the sensitivity of the analytical method now employed. However, the anatoxin levels in the SPATT bags during the present study were also much higher than those reported in Wood et al. (2011; average 0.15, range 0.1–0.28 ng of summed anatoxins g (of Strata-X)$^{-1}$ hr^{-1}). It is plausible that there were more toxins in the river water at the three sites investigated in this study compared to the Waipoua. Reasons for this are unknown and require further investigation, but could be related to growth phase, environmental conditions, or the extent of the blooms upstream (especially at the Mataura and Hutt river sites) of the study sites, which are highly likely to contribute to the amount of toxin in the water column downstream.

Whilst the levels of anatoxin measured in the water samples and the SPATT samples cannot be directly compared (as the SPATT samples accumulate anatoxins over the time period they are deployed and the grab samples measure a discrete point in time), the trends observed between the three sample sets appear consistent. The anatoxin concentrations in water and SPATT samples were higher for the Mataura and Hutt rivers compared to the Cardrona River. Additionally, in general the highest anatoxin concentrations recorded in the water samples also align with the highest values measured in the SPATT samples. In the SPATT samples, the trend of increased anatoxin levels is more apparent as the sampling method accumulates toxins over a period of time and can detect multiple ‘pulses’ of released toxins. This

demonstrates how the two sampling methods work in a complementary fashion, with accurate anatoxin concentrations in the river water being determined using the grab samples, but the SPATT samples providing better information on changes of anatoxin levels over an extended period.

In the Cardrona River no dissolved toxins were detected, suggesting that all toxins in the water column were contained within 'free-floating' *Phormidium* cells. However, *Phormidium* cells were only observed microscopically in three of the 26 samples. Possible explanations for this include: the filaments were broken up into individual cells which are difficult to observe via microscopy, the toxins were bound to organic particles that were filtered out during sample processing, or that some dissolved anatoxins were present, but that these were very low and below the limits of detection of the LC-MS method used in this study.

In the Mataura, the majority of the toxins detected were dissolved, which corresponded to the microscopic analysis where very few *Phormidium* filaments were observed. There was one sample (2245 h; Appendix 3) where a large number of *Phormidium* filaments were observed, suggesting that filament (or mat) detachment events occur sporadically. This is also corroborated by our observations of small to moderate pieces of *Phormidium* mats floating down the river at the study sites. These detached and floating pieces of mats represent a significant risk to recreational users as they could be accidently ingested, and to drinking water supplies, where they could result in the release of pulses of toxins into the water.

In the Hutt River, the majority of the toxins detected were also in the dissolved form, however, *Phormidium* filaments were observed in 79% of the samples, albeit at very low concentrations (< 40 cell mL^{-1}). Detection of intracellular toxins when cell concentrations are this low is unlikely to be achievable with the LC-MS method used in this study.

An important consideration is that the anatoxins detected in the water may, especially in the case of the Mataura and Hutt rivers, have travelled many kilometres down the river making it challenging to relate the concentrations to those in nearby mats or the *Phormidium* cover. At the Mataura site we walked c. 500 m upstream of the SPATT deployment sites and found the *Phormidium* mats were extensive, and an assessment from a road bridge c. 10 km upstream also revealed > 50% *Phormidium* cover. Although the cover was generally isolated to riffles and runs in the Hutt River, extensive coverage was observed at sites for at least 4.5 km upstream of the SPATT deployment site. The Cardrona River site was more isolated as the stream only emerged from the gravel approximately 200 m upstream of the SPATT site, therefore all toxins detected had to have been released in this stream reach. Although we did not characterise toxins or cover across this whole reach, cover was visually similar.

4.3. Relationship to human health guidelines

Toxin levels in the water samples were below the Provisional Maximum Acceptable Values (PMAVs) in the Drinking Water Standards (6 µg L^{-1} for ATX, 2 µg L^{-1} for HTX, http://www.moh.govt.nz/drinkingwater), however, the detection of toxins in the water demonstrates that during mass die-off events much higher levels could potentially be released into the water column, which could exceed the PMAVs. To our knowledge untreated drinking water is not taken from these sites, however the detection of anatoxin in river water highlights the risk of using rivers experiencing *Phormidium* blooms as water supplies. The current recreational guidelines use *Phormidium* cover, rather than toxin concentrations, to define the thresholds. The data in this study will provide further information that can be used to develop risk models which could help in refining and further developing recreational guidelines for benthic cyanobacteria.

4.4. Potential accumulation in and impacts on aquatic organisms

Although this study focuses on gathering data with the aim of developing a human health value in the NPS-FM, given the detection of toxins in all water samples, it is worth considering the risk that continual exposure to low levels of toxins poses to aquatic organisms and the ecological health of waterways in New Zealand. Only a few studies have explored the effect of anatoxins on freshwater organisms worldwide; however, all showed some effect. These include:

- Gilbert (1994) demonstrated that fertility and survival of rotifers decreased when exposed to ATX. This toxicity was enhanced when water temperature was increased and food availability decreased (Gilbert 1996a, 1996b).
- Toad (*Bufo arenarum*) embryos at certain stages of development experienced dose-dependent transient necrosis (cell death), edema (swelling) and loss of equilibrium when exposed to ATX. At the highest dose (30 mg L^{-1}) there were no survivors (Rogers et al. 2005).
- A variety of effects on different developmental stages of fish have been demonstrated. Oberemm et al. (1999) demonstrated that ATX concentrations of 400 µg L^{-1} caused the heart rate of zebrafish to be temporarily altered. Osswald et al. (2007) exposed juvenile carp (*Cyprinus carpio*) to water contaminated with extracts from ATX-producing strains and noted that swimming was altered. Rymuszka and Sieroslawska (2010) showed that ATX is an inducer of apoptosis (programmed cell death) in fish immune cells.
- Mitrovic et al. (2004) provided evidence that ATX has negative effects on the aquatic plants *Lemna minor* and *Cladophora fracta*. Exposure of the plants to 25 µg mL^{-1} increased detoxification processes and resulted in the formation of reactive oxygen species indicating oxidative stress.

No studies have been undertaken in New Zealand to determine if aquatic organisms found within close vicinity to toxin-producing benthic mats bioaccumulate anatoxins. Several recent studies have detected ATX in aquatic organisms. Osswald et al. (2007) placed juvenile carp (*C. carpio*) in water contaminated with extracts of an ATX-producing strain. After 96 hours of exposure, minor levels (0.005–0.073 µg g^{-1}) were found to have accumulated in the fish. In a similar experiment, blue mussels (*Mytilus galloprovincialis*) were exposed to water contaminated with extracts of an ATX-producing strain, and accumulation and depuration were monitored (Osswald et al. 2008). Anatoxin-a was detected in the digestive tract, muscles and foot. One day after beginning the depuration, toxins could not be detected, suggesting it is actively detoxified (Osswald et al. 2008).

As part of the present study we undertook a preliminary assessment of the potential for anatoxin to bioaccumulate in three freshwater species at the Mataura River site: the mayfly *Deleatidium* sp., the freshwater snail *Potamopyrgus antipodarum*, and the flatworm Platyhelminthes. Specimens were maintained in river water for 24 hrs (to purge digestive tracts of food and possible *Phormidium* cells), and washed c. 5 times in Milli-Q water, and analysed using LC-MS for anatoxins. All tested positive for anatoxins. As the data are preliminary and the method is not yet validated we cannot provide actual concentrations or which congeners were present. Based on these preliminary data we strongly recommend that further research is undertaken to assess the effects of anatoxins on a variety of freshwater species, to validate anatoxin extraction methods for use of freshwater species, and to investigate the accumulation of anatoxins in freshwater species, in particular edible taxa such as trout and eels.

4.5. Knowledge gaps and future work

Results from this study suggest that there is some temporal variability in toxin release into river water. Based on these datasets it appears that there may be an increase in toxins during the morning in the Cardrona and Mataura sites, but this pattern was not observed in the Hutt River. Longer-term sampling of mats and water for toxin content and corresponding physiochemical variables may help determine the factors promote toxin release and to identify periods of highest risk. The mechanism of release is currently unknown but could be due to the lysis (breaking open) of *Phormidium* cells and the release of toxins, or the toxins may be actively transported out of the cells which has been suggested for other cyanobacteria species (Pearson et al. 2004).

There appeared to be a relationship between the anatoxin load present in the river (a combination of the anatoxin content of the *Phormidium* mats and the abundance of toxin-containing *Phormidium* mats) and the concentration of anatoxins detected in the water column. To establish a robust relationship between these factors would require further data from more sites which span a wider range of *Phormidium* cover and toxin

concentrations. We suggest that somewhere in the order of 30–50 rivers should be surveyed to provide a robust result. As the long-term sampling conducted during the present study would not be required to evaluate this relationship, this could be achieved relatively easily by developing a standardised sampling protocol and sampling kit. This could be dispatched to regional councils, who could undertake the sampling and would likely involve site surveys, water and mat sampling, and short-term deployments of SPATT samplers.

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6. REFERENCES

APHA 2012. Standard methods for the examination of water and wastewater, 22nd edition. American Public Health Association (APHA), American Water Works Association (AWWA) & Water Environment Federation (WEF).

Brasell K, Heath M, Ryan K, Wood S 2015. Successional change in microbial communities of benthic *Phormidium*-dominated biofilms. Microbial Ecology 69: 254-266.

Cantoral Uriza EA, Asencio AD, Aboal M. 2017. Are we underestimating benthic cyanotoxins? Extensive sampling results from Spain. Toxins 28:9(12).

Fetscher AE, Howard MDA, Stancheva R, Kudela RM, Stein ED, Sutula MA, Busse LB, Sheath RG 2015. Wadeable streams as widespread sources of benthic cyanotoxins in California, USA. Harmful Algae 49: 105-111.

Gilbert JJ 1994. Susceptibility of planktonic rotifers to a toxic strain of *Anabaena flos-aquae*. Limnology and Oceanography 39:1286–1297.

Gilbert JJ 1996a. Effect of temperature on the response of planktonic rotifers to a toxic cyanobacterium. Ecology 77: 1174–80.

Gilbert JJ 1996b. Effect of food availability on the response of planktonic rotifers to a toxic strain of the cyanobacterium *Anabaena flos-aquae*. Limnology and Oceanography 41:1565–1572.

Harland F, Wood SA, Broady P, Williamson W, Gaw S 2014. Polyphasic studies of benthic freshwater cyanobacteria in Canterbury, New Zealand. New Zealand Journal of Botany 52: 116-135.

Hart DD, Biggs BJF, Nikora VI, Flinders CA 2013 Flow effects on periphyton patches and their ecological consequences in a New Zealand river. Freshwater Biology 58: 1588-1602.

Heath M, Wood SA, Ryan K 2010. Polyphasic assessment of fresh-water benthic mat forming cyanobacteria isolated from New Zealand. FEMS Microbiology 73:95-109.

McAllister T, Wood SA, Hawes I 2016. The rise of toxic benthic *Phormidium* proliferations: a review of their taxonomy, distribution, toxin content and factors regulating prevalence and increased severity. Harmful Algae 55:282–294.

Mitrovic SM, Pflugmacher S, James KJ, Furey A 2004. Anatoxin-a elicits an increase in peroxidase and glutathione S-transferase activity in aquatic plants. Aquatic Toxicology 68:185–192.

Oberemm A, Becker J, Codd GA, Steinberg C 1999. Effects of cyanobacterial toxins and aqueous crude extracts of cyanobacteria on the development of fish and amphibians. Environmental Toxicology 14:77–88.

Osswald J, Rellán S, Carvalho AP, Gago A, Vasconcelos V 2007. Acute effect of anatoxin-a producing cyanobacteria on juvenile fish *Cyprinus carpio*. Toxicon 49:693–698.

Osswald J, Rellán S, Gago A, Vasconcelos V 2008. Uptake and depuration of anatoxin-a by the mussel *Mytilus galloprovincialis* (Lamarck, 1819) under laboratory conditions. Chemosphere 72: 235-1241.

Pearson LA, Hisbergues M, Börner T, Dittmann E, Neilan BA 2004. Inactivation of an ABC transporter gene, *mcyH*, results in loss of microcystin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. Applied and Environmental Microbiology 70: 6370-6378.

Puddick J, McGrail I, van Ginkel R, Wood S, Selwood A, Munday R 2017b. 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. Prepared for the New Zealand Ministry for the Environment. Cawthron Report No. 2931. 26 p. plus appendices.

Puddick J, van Ginkel R, Brooke D, Wood S, Selwood A, Truman P, Munday R 2017a. Phormidium Toxicity Interim Report – June 2017. Prepared for the New Zealand Ministry for the Environment. Cawthron Report No. 3032. 33 p. plus appendices.

Rogers EH, Hunter ES, Moser VC, Phillips PM, Herkovitz J, Muñoz L, Hall LL, Chernoff N 2005. Potential developmental toxicity of anatoxin-a, a cyanobacterial toxin. Journal of Applied Toxicology 25: 527–234.

Rymuszka A, Sieroslawska A 2010. Study on apoptotic effects of neurotoxin anatoxin-a on fish immune cells. Neuroendocrinology Letters 31 (Supplement 2): 11-15.

Wood SA, Puddick J 2017. The abundance of toxic genotypes is a key contributor to anatoxin variability in *Phormidium*-dominated benthic mats. Marine Drugs 15(10): 307.

Wood SA, Heath MW, Kuhajek J, Ryan KG 2010. Fine-scale spatial variability of anatoxin-a and homoanatoxin-a production in benthic cyanobacteria; implication for monitoring and management. Journal of Applied Microbial Ecology 109:2011-2018.

Wood SA, Holland PT, MacKenzie LA 2011. Development of solid phase adsorption toxin tracking (SPATT) for monitoring anatoxin-a and homoanatoxin-a in river water. Chemosphere 82: 888-894.

Wood SA, Smith FMJ, Heath MW, Palfroy T, Gaw S, Young R, Ryan KG 2012. Within-mat variability in anatoxin-a and homoanatoxin a production among benthic *Phormidium* (Cyanobacteria) strains. Toxins 4:900-912.

Wood S, Hawes I, McBride G, Truman P, Dietrich D 2015a. Advice to inform the development of a benthic cyanobacteria attribute. Prepared for Ministry for the Environment. Cawthron Report No. 2752. 91 p.

Wood SA, Depree C, Brown L, McAllister T, Hawes I 2015b. Entrapped sediments as a source of phosphorus in epilithic cyanobacterial proliferations in low nutrient rivers. PloS One 10(10):e0141063.

Wood SA, Puddick J, Fleming R, Heussner AH 2016. Detection of anatoxin-producing Phormidium in a New Zealand farm pond and an associated dog death. New Zealand Journal of Botany 55:1-11.

Wood SA, Atalah J, Wagenhoff A, Doehring K, Hawes I 2017. Investigating environmental drivers of Phormidium blooms. Prepared for Ministry for the Environment. Cawthron Report No. 2956. 77 p. plus appendix.

7. APPENDICES

Appendix 1. Summed and individual anatoxin congeners (anatoxin-a (ATX), homoanatoxin-a (HTX), dihydro-anatoxin-a (dhATX), and dihydro-homoanatoxin-a (dhHTX)) concentrations in 15 *Phormidium* mat samples collected from the Cardrona River (A), Mataura River (C) and Hutt River (E) on 11 and 12 April, and 29 November 2017, respectively. B, D and F are the percentage of each congener in the mat samples.

A.

	ATX	HTX	dhATX	dhHTX	Sum
	mg/kg/dw				
1	0.05	2.23	9.37	2.28	13.93
2	0.09	5.69	28.02	7.95	41.76
3	0.04	3.53	24.19	19.46	47.21
4	0.05	1.52	12.77	1.40	15.73
5	0.07	0.98	3.13	0.23	4.41
6	0.42	18.64	213.61	6.52	239.18
7	0.10	5.97	41.78	23.90	71.75
8	0.06	0.39	1.54	0.20	2.18
9	0.03	1.55	11.67	6.19	19.44
10	0.00	0.58	3.62	1.12	5.32
11	0.00	2.20	17.58	7.14	26.91
12	0.04	0.91	6.24	2.75	9.93
13	0.02	1.28	13.68	7.16	22.14
14	0.00	3.05	46.41	30.84	80.30
15	0.02	0.39	1.44	0.21	2.07

B.

	ATX	HTX	dhATX	dhHTX
	% of summed anatoxins			
1	0%	16%	67%	16%
2	0%	14%	67%	19%
3	0%	7%	51%	41%
4	0%	10%	81%	9%
5	2%	22%	71%	5%
6	0%	8%	89%	3%
7	0%	8%	58%	33%
8	3%	18%	71%	9%
9	0%	8%	60%	32%
10	0%	11%	68%	21%
11	0%	8%	65%	27%
12	0%	9%	63%	28%
13	0%	6%	62%	32%
14	0%	4%	58%	38%
15	1%	19%	70%	10%

C.

	ATX	HTX	dhATX	dhHTX	Sum
	mg/kg/dw				
1	0.16	0.51	4.61	5.07	10.34
2	0.85	2.61	196.97	266.67	467.09
3	0.34	1.45	34.21	43.42	79.42
4	0.38	5.00	26.19	116.67	148.24
5	0.45	7.11	419.32	852.27	1279.16
6	0.22	1.33	546.91	148.15	696.62
7	0.69	1.13	577.08	109.38	688.27
8	1.07	2.52	85.07	131.34	220.01
9	0.17	0.43	47.31	25.81	73.72
10	0.67	1.64	170.00	221.11	393.42
11	0.46	2.71	166.12	368.98	538.27
12	0.70	4.57	142.76	532.89	680.92
13	0.88	1.77	139.42	161.31	303.37
14	3.53	10.43	619.15	1051.06	1684.17
15	1.94	3.62	172.64	280.19	458.40

D.

	ATX	HTX	dhATX	dhHTX
	% of summed anatoxins			
1	2%	5%	45%	49%
2	0%	1%	42%	57%
3	0%	2%	43%	55%
4	0%	3%	18%	79%
5	0%	1%	33%	67%
6	0%	0%	79%	21%
7	0%	0%	84%	16%
8	0%	1%	39%	60%
9	0%	1%	64%	35%
10	0%	0%	43%	56%
11	0%	1%	31%	69%
12	0%	1%	21%	78%
13	0%	1%	46%	53%
14	0%	1%	37%	62%
15	0%	1%	38%	61%

Appendix 1 cont.

E.

	ATX	HTX	dhATX	dhHTX	Sum
	mg/kg/dw				
1	0.39	7.36	12.56	2.21	22.52
2	5.00	176.54	300.00	11.54	493.08
3	1.57	76.86	97.65	20.39	196.47
4	1.19	41.58	104.95	13.47	161.19
5	0.18	4.62	7.20	0.20	12.20
6	14.37	314.95	613.20	47.38	989.90
7	9.02	320.78	339.22	93.33	762.35
8	6.48	223.24	488.76	67.43	785.90
9	3.36	148.79	274.39	56.82	483.36
10	0.19	2.59	11.91	0.39	15.07
11	0.26	8.02	12.04	0.99	21.31
12	6.97	178.72	441.47	20.92	648.07
13	17.52	1029.33	920.38	148.95	2116.19
14	14.10	731.81	687.62	151.62	1585.14
15	0.43	12.49	18.73	1.53	33.18

F.

	ATX	HTX	dhATX	dhHTX
	% of summed anatoxins			
1	2%	33%	56%	10%
2	1%	36%	61%	2%
3	1%	39%	50%	10%
4	1%	26%	65%	8%
5	1%	38%	59%	2%
6	1%	32%	62%	5%
7	1%	42%	44%	12%
8	1%	28%	62%	9%
9	1%	31%	57%	12%
10	1%	17%	79%	3%
11	1%	38%	56%	5%
12	1%	28%	68%	3%
13	1%	49%	43%	7%
14	1%	46%	43%	10%
15	1%	38%	56%	5%

Appendix 2. Tukey HSD post-hoc pair-wise test results for the (A) Mataura River (12 and 13 April 2017) and (B) Hutt River (28 and 29 November 2017) water total anatoxin results. Significant results ($P < 0.05$) are shown. Data from the Cardrona River was not assessed as no significant differences were detected by one-way ANOVA.

A

Sampling time	1045	1245	1445	1645	1845	2045	2245	1245	345	645	845	1045	1245
1045													
1245	0.01												
1445	0.01	0.01											
1645	0.01	0.01											
1845	0.01	0.01											
2045	0.01	0.01											
2245	0.01	0.01											
1245		0.01	0.01	0.01	0.01								
345		0.01											
645		0.01											
845	0.01	0.01						0.01	0.01	0.01			
1045	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01		
1245	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	

B

Sampling time	1000	1200	1400	1630	1830	2030	2200	0000	0200	0400	0600	0800	1000
1000													
1200													
1400													
1630													
1830													
2030		0.05											
2200		0.01	0.05										
0000													
0200							0.05						
0400							0.05						
0600				0.05	0.05		0.05						
0800				0.05	0.05		0.05						
1000				0.05	0.05		0.05						

Appendix 3. *Phormidium* cell concentrations in water samples collected from the Cardrona (10 and 11 April 2017), Mataura (12 and 13 April 2017) and Hutt (28–29 November 2017) rivers. Where cells were observed in more than one triplicate data are averages (± standard deviation). Only samples where cells were observed are given.

Cardrona River		Mataura River		Hutt River	
Time	Cell conc. (cell mL^{-1})	Time	Cell conc. (cell mL^{-1})	Time	Cell conc. (cell mL^{-1})
1310	17.1	1245	2.1	0000	0 (±0)
1710	28.1	1445	3.6	1200	12.7 (±11.3)
2310	17.3	1645	4.3	1400	39.8 (±6.9)
		1645	7.9	1630	13.8 (±5.8)
		2045	12.6	1830	13.1 (±7.1)
		2045	3.5	2030	21.7 (±11.1)
		2245	33.0	2200	14.4 (±5.5)
		2245	2.9	0000	10.8 (±11.1)
				0200	2.2 (±2.8)
				0400	10.7 (±10.5)
				0600	7.3 (±9.7)
				0800	9.5 (±0.2)
				1000	14.7 (±6.1)

Appendix 4. Tukey HSD post-hoc pair-wise test results for the (A) Cardrona River, (B) Mataura River and (C) Hutt River for SPATT bag anatoxin results. Significant results (P < 0.05) are shown.

A. Cardrona River

Sampling time	1510	1710	1910	2110	2310	110	310	510	710	910	1110	1310
1510												
1710												
1910												
2110												
2310												
0110												
0310												
0510						0.05	0.05					
0710	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01				
0910	0.05	0.01	0.01	0.01	0.01	0.01	0.01		0.01			
1110	0.05	0.01	0.01	0.01	0.01	0.01	0.01		0.01			
1310									0.01			

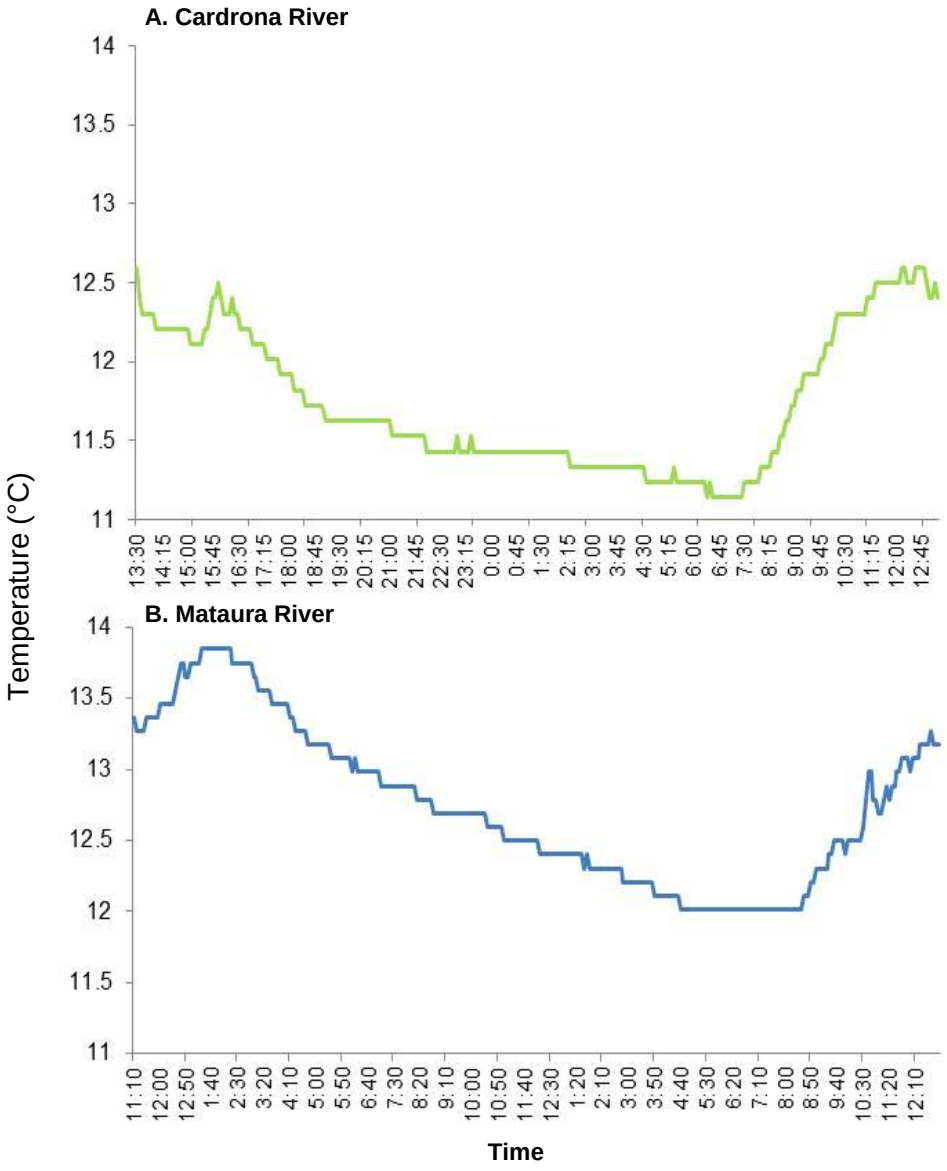
B. Mataura River

Sampling time	1245	1445	1645	1845	2045	2245	1245	345	645	845	1045	1245
1245												
1445												
1645												
1845												
2045												
2245												
1245												
345												
645												
845												
1045				0.05	0.05	0.05	0.05	0.05				
1245												

C. Hutt River

Sampling time	1200	1400	1630	1830	2000	2200	000	200	400	600	800	1000
1200												
1400												
1630												
1830	0.05	0.05	0.05									
2000	0.01	0.01	0.01									
2200	0.01	0.01	0.01									
000	0.01	0.01	0.01									
200					0.01	0.05						
400	0.05				0.01	0.05						
600	0.05					0.05						
800	0.01	0.01	0.01									
1000	0.01	0.01	0.01									

Appendix 5. Temperature measured every 5 min over the sampling period in the (A) Cardrona River (10–11 April 2017) and (B).Mataura River (12–13 April 2017). Note: temperature loggers were not deployed at the Hutt River.



Appendix 6. Physiochemical parameters measured over the sampling period in the Cardrona River, Mataura River and Hutt River.

Cardrona River							Matuara River							Hutt River						
Date	Time	Diss. O^2 (%)	Cond. (µS cm^{-1})	pH	DIN (mg L^{-1})	DRP (mg L^{-1})	Date	Time	Diss. O^2 (%)	Cond. (µS cm^{-1})	pH	DIN (mg L^{-1})	DRP (mg L^{-1})	Date	Time	Diss. O^2 (%)	Cond. (µS cm^{-1})	Temp (˚C)	DIN (mg L^{-1})	DRP (mg L^{-1})
10 Apr 17	1310		108.70	7.56	0.136	< 0.004	12 Apr 17	1045	102.30	113.8	7.26	0.504	0.006	28 Nov 17	1000	107.5	110.2	19.2	0.180	< 0.004
10 Apr 17	1510	73.6	108.30	7.18	0.215	< 0.004	12 Apr 17	1245	114.80	114	7.98	0.684	0.006	28 Nov 17	1200	109.7	110.3	21	0.166	< 0.004
10 Apr 17	1710	73.2	108.30	7.01	0.401	< 0.004	12 Apr 17	1445	119.80	114.4	8.28	0.100	0.005	28 Nov 17	1400	111.6	110.4	22.1	0.148	< 0.004
10 Apr 17	1910	73.2	108.40	7.05	0.295	< 0.004	12 Apr 17	1645	110.20	115.8	7.79	0.782	0.010	28 Nov 17	1630				0.087	< 0.004
10 Apr 17	2110	73.9	108.70	7.02	0.355	< 0.004	12 Apr 17	1845	100.90	116.6	7.47	0.711	0.005	28 Nov 17	1830	103.8	105.4	22.6	0.146	< 0.004
10 Apr 17	2310	72.1	108.40	7.1	0.435	< 0.004	12 Apr 17	2045	95.40	118	7.41	0.564	0.009	28 Nov 17	2030	94.4	105.3	21.5	0.119	< 0.004
11 Apr 17	0110	71.3	108.50	7.2	0.385	0.004	12 Apr 17	2245	93.80	119.3	7.31	0.714	0.008	28 Nov 17	2200	88.7	105.6	20.5	0.058	< 0.004
11 Apr 17	0310	71.4	107.40	7.15	0.405	< 0.004	12 Apr 17	1245	91.60	119.6	7.31	0.572	0.006	29 Nov 17	0000	86.8	105.7	19.9	0.210	< 0.004
11 Apr 17	0510	73.1	108.60	7.15	0.464	< 0.004	13 Apr 17	0345	90.50	120.9	7.2	0.639	0.007	29 Nov 17	0200	86.9	106	19.2	0.104	< 0.004
11 Apr 17	0710	71.1	107.00	7.25	0.176	< 0.004	13 Apr 17	0645	89.10	123.9	7.29	1.114	0.011	29 Nov 17	0400	86	107.8	18	0.244	< 0.004
11 Apr 17	0910	76.4	106.00	7.24	0.183	< 0.004	13 Apr 17	0845	99.00	123	7.26	0.306	0.006	29 Nov 17	0610	86	107.1	17.9	0.143	< 0.004
11 Apr 17	1110	79.2	108.40	7.16	0.403	< 0.004	13 Apr 17	1045	112.40	126.2	7.57	0.355	0.006	29 Nov 17	0800	89.2	106.9	17.9	0.107	< 0.004
11 Apr 17	1310	71.7	108.60	7.01	0.355	< 0.004	13 Apr 17	1245	121.60	132	7.51	0.458	0.011	29 Nov 17	1000	100.6	104.5	18.6	0.042	< 0.004