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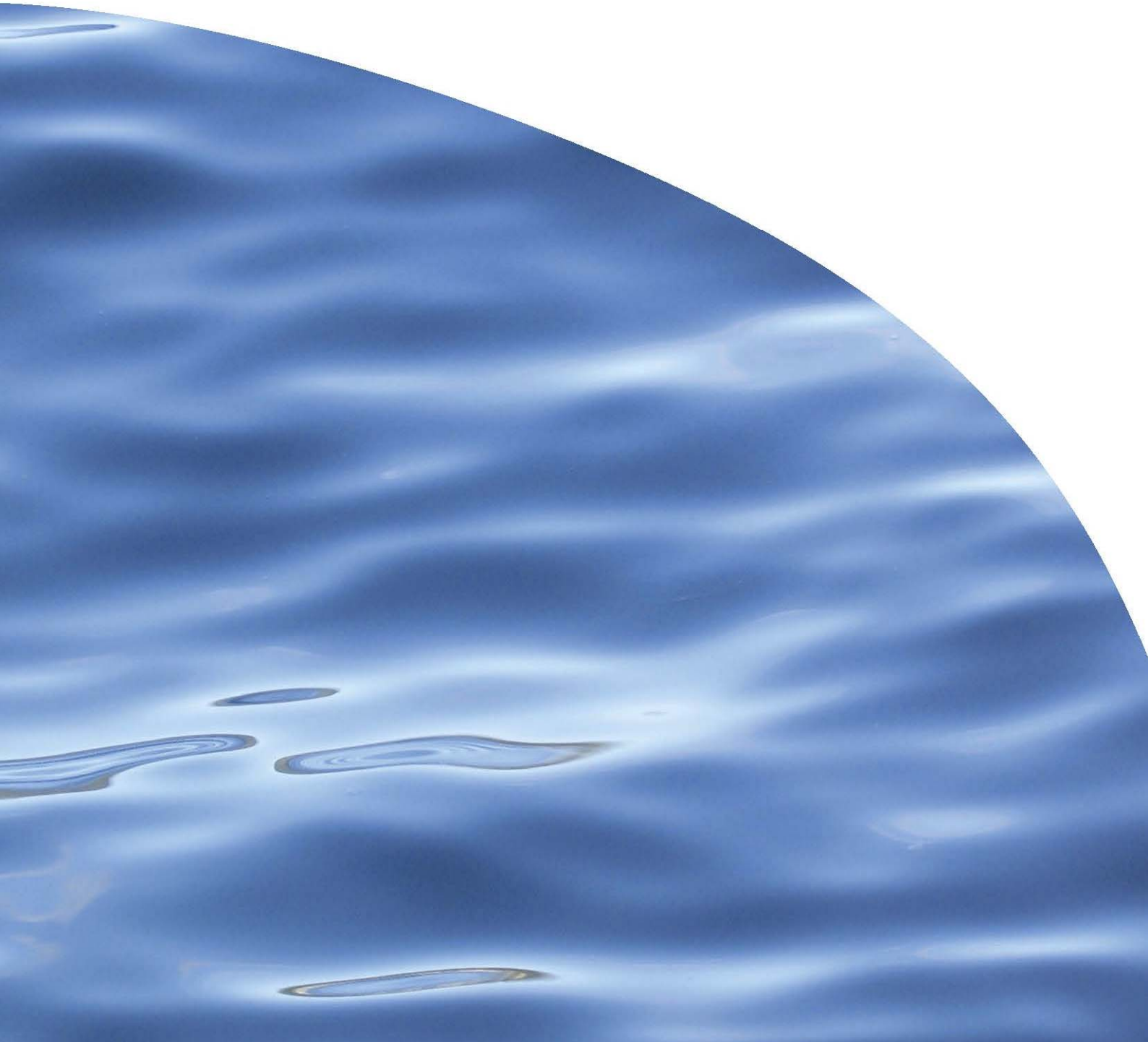

THE EFFECT OF RIVER FLOW AND NUTRIENTS ON *PHORMIDIUM* ABUNDANCE AND TOXIN PRODUCTION IN RIVERS IN THE MANAWATU-WHANGANUI REGION

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Prepared for Horizons Regional Council

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EXECUTIVE SUMMARY

Phormidium blooms (defined as greater than 20% mean percentage coverage) have been notable in multiple rivers in the Manawatu-Whanganui region since cyanobacterial-specific monitoring (as opposed to general periphyton monitoring where cyanobacteria were not separated from 'mats') begun in 2011. This is of particular concern in rivers that are used as drinking water sources or for recreational activities as *Phormidium* produces four potent neurotoxins; anatoxin-a, homoanatoxin-a, dihydro-anatoxin-a and dihydro-homoanatoxin-a. In 2011, a study of rivers in the Manawatu-Whanganui region found variable *Phormidium* coverage and toxin concentrations among 14 sites (Wood & Young 2011). One of the recommendations from the 2011 study was to investigate the influence of physico-chemical parameters on *Phormidium* abundance by monitoring selected sites weekly over an extended period. Based on these recommendations Horizons Regional Council selected 10 sites in seven rivers and undertook weekly monitoring from January 2012 to June 2013.

Phormidium mats were present at all 10 sampling sites in variable abundances during the survey. These five sites had mean *Phormidium* coverage greater than 40% on multiple sampling occasions:

1. Makakahi River at Hamua
2. Manawatu River downstream of the Palmerston North City Council (PNCC) sewage treatment plant (STP)
3. Mangatainoka River at State Highway 2 (SH2)
4. Oroua River downstream of Feilding STP
5. Tokomaru River at Horseshoe Bend)

There were three sites (Oroua River upstream of Feilding STP, Oruakeretaki River at SH2, and Tiraumea River at Ngaturi) where mean *Phormidium* cover never exceeded 15%, and two sites (Manawatu River upstream of PNCC STP, and Mangatainoka River at Putara) where mean *Phormidium* cover never exceeded 2%.

Multiple physico-chemical factors interact and influence *Phormidium* blooms and there is a hierarchy in importance among these and this differs among rivers / sites. Among the five sites with *Phormidium* blooms, hydrologic disturbance was identified as a key factor in regulating coverage. The intensity of flushing flow required to maintain or reduce *Phormidium* mat cover to below 20% for 90% of the time varied markedly among sites. The Manawatu River downstream of PNCC STP site required the lowest 'times median flow' (0.65). In contrast a flow 5.35 'times median flow' would be required at Mangatainoka River at SH2. There was insufficient data to use this approach at the Oroua River downstream of the Feilding STP.

Flushing flows do not explain why some rivers (or sites within a river) experience blooms and others do not. This study and other research has identified the importance of nutrient concentrations and suspended fine sediment. As *Phormidium* mats become established they

create internal conditions that facilitate phosphorus release from incorporated sediments. As such, the most time-critical period for nutrients in the water column to influence *Phormidium* is during the initial stage of mat formation. Our analysis suggests that a level of dissolved reactive phosphorus (DRP) in the water column of less than 0.01 mg/L will promote *Phormidium* blooms. There were two sites with blooms where DRP was above this (ca. 0.02 mg/L), and both were downstream of STPs and further investigation is required. Analysis of a larger dataset (45 periphyton sampling sites in the Manawatu region), provided further evidence to support the observation of blooms occurring when DRP was less than 0.01 mg/L, except at sites downstream of STP.

A dissolved inorganic nitrogen (DIN) water column concentration of greater than 0.20 mg/L promoted *Phormidium* blooms at four of the five sites studied. This corroborates early studies that have shown *Phormidium* isolated from New Zealand rivers cannot fix nitrogen, and that elevated DIN promotes growth (Heath *et al*. 2014a). The DIN concentrations at the Tokomaru River at Horseshoe Bend site were below this threshold; however, this site experiences prolonged *Phormidium* blooms. *Phormidium* co-occurs in mats with other micro-organisms (*e.g.* bacteria, diatoms).Preliminary analysis indicates that any nitrogen-fixing species present within mats, may be a nitrogen source for *Phormidium*. Additionally, a significant flow (ca. 16.5 times the long-term median) is required to reduce mat coverage to less than 20% at this site. This would provide longer, stable periods for mats to establish, or result in a larger starting inoculum following a flushing event. These ‘small batches of mats’ may also already contain other nitrogen-fixing organisms.

Total anatoxin concentrations and the relative composition of structural variants varied spatially and temporally among and within rivers. A maximum concentration of 82.4 mg/kg freeze-dried weight was detected on one occasion. There were three sites where no toxins were detected. There was no correlation between the concentrations of toxins and *Phormidium* coverage, or any of the physico-chemical variants measured. We suggest the variability in toxin concentration is largely due to differences in the relative abundance of toxic and non-toxic genotypes within each mat.

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1. INTRODUCTION

The filamentous cyanobacterial genus *Phormidium* is found worldwide in diverse habitats ranging from intertidal marshes to Arctic and Antarctic lakes (Komárek & Anagnostidis 2005). Some *Phormidium* species form benthic mats under favourable conditions. In New Zealand there has been an apparent increase in the prevalence of benthic *Phormidium* mats in some rivers. Heath *et al*. (2010) used molecular techniques to demonstrate that *Phormidium autumnale (Ph. autumnale)* was the species responsible for blooms in New Zealand rivers. When hydrological and environmental conditions are suitable, *Ph. autumnale* can form thick cohesive black / brown mats that cover extensive areas of the river substrate. During routine site surveys, all mats with this distinct macroscopic morphology are assumed to be dominated by *Ph. autumnale* (hereafter referred to as *Phormidium*).

Phormidium produces powerful neuromuscular-blocking toxins, collectively known as anatoxins. These toxins pose a threat to humans and animals when consumed or when there is contact with contaminated water. Anatoxins have killed approximately 100 dogs in New Zealand in the last five years and resulted in health warnings against any contact with the water having been posted along the banks of many rivers. Recent studies also indicate high toxicity of aqueous *Phormidium* extracts to the mayfly *Deleatidium* (Wood & Bridge unpub. data). However, the impact of anatoxins and *Phormidium* mats on aquatic communities is largely unknown.

There are four chemical structural variants of anatoxins; anatoxin-a (ATX), homoanatoxin-a (HTX) and the reduced derivatives dihydro-anatoxin-a (dhATX) and dihydro-homoanatoxin-a (dhHTX). In *Phormidium* blooms, ATX, HTX, dhATX and dhHTX are almost always detected simultaneously, however, their relative concentrations vary. Dihydro-anatoxin-a and dhHTX have an approximately 10-fold reduction in their affinity for nicotinic acetycholine binding sites in comparison to ATX and HTX (Wonnacott *et al.* 1991); thus it is assumed they are 10 times less toxic. Knowledge on which variants are present, and how these vary in an environmental context, may help in refining risk assessments.

Phormidium prevalence has been notable in multiple rivers in the Manawatu-Whanganui region since cyanobacterial-specific (as opposed to general periphyton monitoring where cyanobacteria were not separated from 'mats') monitoring begun in 2011 (Wood & Young 2011, 2012). In some cases the presence of *Phormidium* has led to closure of swimming areas in accordance with the New Zealand Guidelines for Cyanobacteria in Recreational Freshwaters (Ministry for the Environment and Ministry of Health 2009). *Phormidium* is also prevalent in the vicinity of some public water supply intakes.

Horizons Regional Council (Horizons) has been collecting *Phormidium* coverage, toxin and related physico-chemical data weekly at 10 sites for 12 to 18 months.

Preliminary analysis of the first six months of these data indicated that *Phormidium* blooms generally occur in rivers with very low dissolved reactive phosphorus (DRP; ca. < 0.01 mg/L) and elevated dissolved inorganic nitrogen (DIN; ca. > 0.1 mg/L; Wood & Young 2012). Similar nutrient thresholds have been identified in other New Zealand rivers with *Phormidium* blooms *e.g.* Hutt River (Wellington; Heath *et al*. 2013) and Maitai River (Nelson; Wood & Bridge 2014). Molecular techniques have shown that cultures of *Phormidium* isolated from New Zealand rivers cannot fix nitrogen and these data collaborate the initial suggestion, based on the Horizons dataset, that increased DIN may be required before *Phormidium* will bloom.

The frequency of flushing flows has been cited as the most important factor in determining whether a *Phormidium* bloom will be present at sites where proliferations are known to occur (Heath *et al*. 2014b). During flushing flows, *Phormidium* mats are removed by elevated river flows, which cause high shear stresses, abrasion by mobilised sediments, and movement of the substrata (Clausen & Biggs 1997, Biggs, *et al*. 1999, Francoeur & Biggs 2006). A flow event larger than three times the long-term median flow is widely given as the flow required to remove periphyton in New Zealand rivers (Clausen & Biggs 1997, Milne & Watts 2007). Preliminary analysis of the first six months of the Horizons dataset indicated that this 'rule-of-thumb' may not be applicable at all sites (Wood & Young 2012). Wood and Young (2012) suggested this may be due to differing stages of *Phormidium* mat development, with recently-established mats having stronger adhesion to the substrate, compared to well-developed thick mats. They also postulated that rivers with a low median flow may need a flush greater than three times the median to generate a sufficient velocity to remove mats or mobilise sediment (Wood & Young 2012).

Initial analysis of the six month Manawatu-Whanganui dataset showed marked temporal variations in the concentrations of anatoxins. However, there was no correlation between toxin concentrations and percentage *Phormidium* coverage, or any of the environmental parameters measured. It is unknown whether this variability is due to shifts in the relative abundance of toxic and non-toxic genotypes that co-occur in mats, or whether biotic / abiotic parameters triggers increased production of toxins. Analysis of a larger dataset may provide new insights or may offer further evidence to confirm the initial observations.

The objectives of this project were to undertaken a thorough analysis of the full 12 to 18 months of data from the Manawatu-Whanganui region to:

- investigate the intensity of flushing flow required to remove *Phormidium* mats and establish whether a single metric can be used for all rivers studied.
- explore whether thresholds in DRP and DIN that render a river more likely to experience *Phormidium* blooms in the Manawatu-Whanganui region can be identified.

- establish if there are any correlations between anatoxin production (and variants produced) and environmental factors.

2. METHODS

2.1. Sample locations

One or two sites at each of seven rivers (Makakahi, Manawatu, Mangatainoka, Oroua, Oruakeretaki, Tiraumea, and Tokomaru) were selected for benthic cyanobacterial monitoring (Figure 1). Sites were selected based on sampling in 2011 (Wood & Young 2011), the review of the initial six months of data (Wood & Young 2012), and chosen to represent rivers with and without historical cyanobacterial proliferations and with a range of nutrient and flow regimes. Sampling and surveying was undertaken approximately weekly at seven sites between 6 January 2012 to 26 or 28 June 2013. The Manawatu River upstream and downstream of the Palmerston North City Council sewage treatment plant (PNCC STP) sites were monitored from 18 July 2012 to 28 June 2013 and the Oruakeretaki River at State Highway Two (SH2) from 17 August 2012 to 28 June 2013.

2.2. Site surveys

All site surveys and sampling were undertaken by Horizons staff. The transect method, outlined in the New Zealand Guidelines for Managing Cyanobacteria in Recreational Fresh Waters (Ministry for the Environment and Ministry of Health 2009), was used to determine the percentage of the river substrate covered by *Phormidium* mats.

The surveys conducted in this study were based in stream / river runs and riffles. The length of the reach surveyed varied from 30 m to100 m. At each site, four transects at right angles to the water's edge and going out to a depth of 0.6 m were surveyed. The *Phormidium* mat cover was assessed at five points along each transect using an underwater viewer. The median of 20 data points was used to visualise temporal variability in the overall *Phormidium* percentage mat cover at each site. The mean of the 20 data points was used for all other analysis. The presence of detaching and exposed mats was also recorded for each transect.

The percentage cover of substrate size classes was determined visually using a single transect on each sampling day and the following categories; macrophytes, moss, mud (< 0.2 mm); sand (0.2 mm–2 mm); fine gravel (2 mm–8 mm); gravel (8 mm–64 mm); cobble (64 mm–264 mm); boulder (> 264 mm) and bedrock. The means were calculated for the entire sampling period.

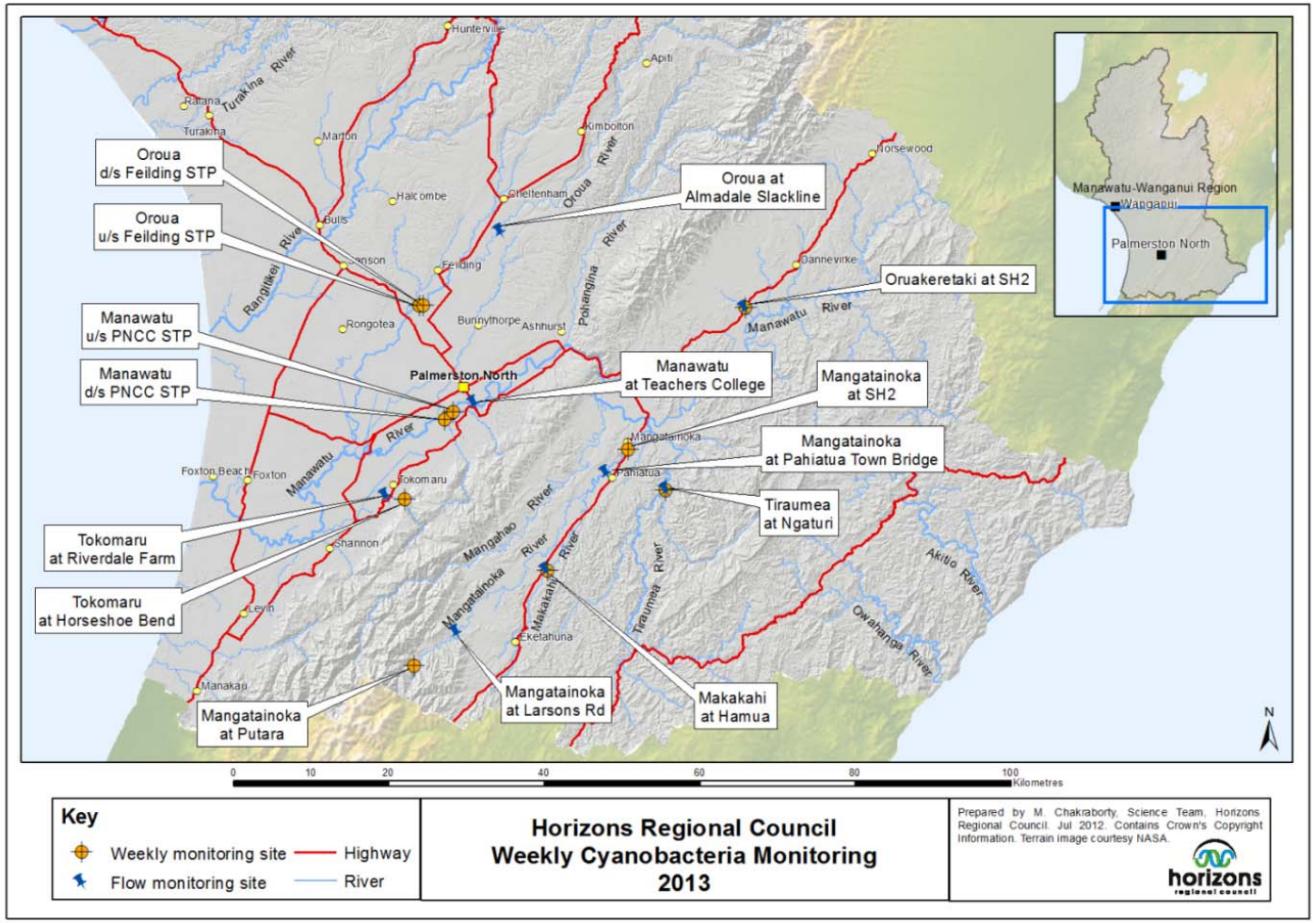

Figure 1. Location of sampling and flow monitoring sites in the Manawatu-Whanganui region, New Zealand. STP = sewage treatment plant, SH2 = State Highway 2, d/s = downstream, u/s = upstream, PNCC = Palmerston North City Council.

2.3. Cyanobacterial sample collection and preparation

A single sample was collected from 10 rocks at each site by a scraping of *Phormidium* mat material from a defined circle with a 6.25 cm diameter. These 10 samples from each site were pooled and placed into a plastic container. The samples were chilled and stored in darkness and transported to Horizons Regional Council, where they were frozen (-20°C). They were later sent (still frozen) to Cawthron Institute (Cawthron) for analyses.

2.4. Toxin extraction and analysis

Phormidium mat samples from each site were pooled, then lyophilised (freeze-dried) and homogenised. Lyophilised material (100 mg) was suspended in 10 mL of Milli-Q water (MQ) containing 0.1 % formic acid and sonicated (Cole Parmer 8890, Biolab, Auckland, New Zealand) for 20 minutes. Samples were then centrifuged (3,000 × *g*, 10 minutes). The supernatants were analysed for anatoxin-a (ATX), homoanatoxin-a (HTX), dihydro-anatoxin-a (dhATX) and dihydro-homoanatoxin-a (dhHTX) using liquid chromatography-mass spectrometry (LC-MS) as described in Heath *et al*. (2010). There are no commercially-available standards for dhATX, HTX, and dhHTX, so concentrations were calculated using the anatoxin-a calibration curve.

2.5. Water temperature, conductivity and river flow

Water temperature, conductivity and pH were measured at each sampling site using a handheld multi-parameter PCTESTR 35 meter.

River flow was measured at state of the environment (SOE) flow recording sites (Figure 1) and in accordance with Horizons ISO 1000-1 and 1100-2 system (Roygard *et al*. 2011). The daily mean flow was determined for each site. Long-term median flows were also used in data analysis (Appendix 1).

2.6. Nutrient data

Water samples were collected weekly to determine nitrite-N, nitrate-N, ammoniacal-N, total nitrogen (TN), total organic nitrogen (TON), dissolved reactive phosphorus (DRP) and total phosphorus (TP) at every site. Samples were analysed by Watercare Laboratory Services.

2.7. Data analysis

Data analysis was undertaken in R 2.12.1 (R Development Core Team, 2010) and Excel (Microsoft).

2.7.1. Relationships between Phormidium coverage and river flow

This analysis was only undertaken for the five sites that had *Phormidium* coverage greater than 20% on multiple occasions; Makakahi River at Hamua, Manawatu River downstream of the PNCC STP, Mangatainoka River at SH2, Oroua River downstream Feilding STP and Tokomaru River at Horseshoe Bend.

For each site, the maximum daily mean flow was determined on the actual day, or the six days prior to surveying. A timeframe of seven days was selected as this is the length of time that *Phormidium* coverage has been observed to go from 0% to greater than 20% cover (Heath *et al*. 2011, Wood & Young 2012). This 'accrual period' will vary among sites and will be dependent on other factors such as:

- initial inoculums *i.e.* amount of material remaining after the last flushing event.
- temperature—with higher temperatures promoting growth (*e.g.* Tang & Vincent 1999).
- photosynthetic active radiation—with higher levels generally increasing growth (*e.g.* Müllner & Schagerl 2003).

Greater than 20% mean coverage of *Phormidium* was selected as this is the lower threshold of the amber alert level in the New Zealand Guidelines for Cyanobacteria in Recreational Freshwater (Ministry for the Environment & Ministry of Health 2009). Additionally, if a site has a mean coverage of 20%, it is likely this will continue to increase in the absence of a flushing event.

A quantile regression approach was used to determine the river flow when 80%, 85% or 90% of the sampling points had *Phormidium* coverage below 20%. Standard linear regression fits a line through the mean response across a given range of predictor values; whereas quantile regression fits a line through the median or any other quantile. It is a useful way to characterise the potential range, or high and low bounds, of a response (Cade & Noon 2003). This is an appropriate approach in this context as the regression line generated indicates the maximum extent of *Phormidium* cover possible, when all other requirements of growth are met. In this dataset we observe many occasions where the predicted potential cover was not met due to some unmeasured limiting factor. However, the measured flow can be used to estimate the maximum cover that could be reached.

The relationship between flow rate and *Phormidium* cover was not linear. Logit transformation is often appropriate to percentage or proportion data, and for this data

was found to improve the performance of the model. Log-transformation of the flow data further improved the model fit. Regression lines were fitted to the models at 80th, 85th and 90th percentiles. Quantile regression analyses was performed in R 2.12.1 (R Development Core Team, 2010), using the *quantreg* package (Koenker 2012).

2.7.2. Relationships with nutrient concentrations

Biggs and Close (1989) suggest simple point-by-point correlations between nutrient concentrations and periphyton biomass do not provide a true indication of the historic nutrient loading on the community. This is because nutrients are progressively depleted from the water column as biomass accrues during the growth cycle. To limit this effect in the current analysis, the cumulative mean of the DIN and DRP concentrations over the accrual period were calculated for each time-point (*i.e.* the mean nutrient concentrations since the previous sampling point where *Phormidium* coverage was zero).

- Data points where a flushing flow may have impacted *Phormidium* coverage were differentiated for graphing. To do this, the maximum daily mean flow was determined for the actual day, or the six days prior to site surveying, was determined. For sites with *Phormidium* coverage less than 20% a sampling point as deemed to be flow-effected if it this exceeded the three times long-term median flow. For sites with coverage greater than 20% on multiple occasions the 90th percentile (or 85% for Tokomaru River at Horseshoe Bend) was used (Figures 12 and 13).

A dataset of 45 other rivers in the Manawatu region was analysed to further investigate the relationship between *Phormidium* coverage and DRP and DIN. This data was collected monthly from 2011 to 2012 and the mean DIN and DRP compared to the maximum *Phormidium* coverage.

Linear regression was used to investigate relationships between water temperature and the mean percentage *Phormidium* coverage.

2.7.3. Relationships between toxin concentration and percentage coverage and environmental parameters

Linear regression was used to investigate relationships between the total anatoxin concentrations (= ATX + HTX + dhATX + dhHTX) and the mean percentage of *Phormidium* coverage, temperature, DIN and DRP **at the time** of sampling.

3. RESULTS

3.1. Abundance and temporal variability in *Phormidium* mats

Phormidium mats were present in variable abundances at some time-points in all 10 sampling sites (Table 1). Five sites had mean *Phormidium* coverage greater than 40% on multiple sampling occasions (Table 1; Figures 2, 4, 6 and 8). Although three sites (Oroua River upstream of Feilding STP, Oruakeretaki River at SH2 and Tiraumea River at Ngaturi) had high *Phormidium* percentage coverage (40% to 60%) in a single view, the mean cover did not exceed 15% (Table 1, Figures 9, 10, 11). At two sites (Manawatu River upstream of PNCC STP and Mangatainoka River at Putara) the mean *Phormidium* coverage did not exceed 2%.

Brief descriptions of temporal variations in *Phormidium* percentage coverage and toxin concentrations at each site are given below.

Table 1. Summary of number of site surveys undertaken and samples collected, *Phormidium* mat coverage and toxins detected at the 10 sampling sites. SH2 = State Highway Two, u/s = upstream, d/s = downstream, PNCC = Palmerston North City Council, STP = sewage treatment plant.

Site	No. of surveys	No. of samples	% of surveys with *Phormidium* present	Max. *Phormidium* % coverage in any one view	Max. mean % across 20 views	% of mats with ana-toxins	Max. anatoxin (mg/kg)
Makakahi River at Hamua	78	31	69	90	47	81	82.4
Manawatu River u/s PNCC STP	50	2	14	5	2	0	-
Manawatu River d/s PNCC STP	50	20	52	90	47	45	0.3
Mangatainoka River at Putara	78	0	18	10	1.5	-	-
Mangatainoka River at SH2	78	47	76	95	56	83	16.6
Oroua River u/s Feilding STP	78	8	19	60	11	0	-
Oroua River d/s Feilding STP	78	11	33	90	45	0	-
Oruakeretaki River at SH2	46	16	63	40	14	94	0.9
Tiraumea River at Ngaturi	78	19	36	50	15	32	0.2
Tokomaru River at Horseshoe Bend	78	29	77	95	74	90	6.2

3.1.1. Makakahi River at Hamua

Median *Phormidium* coverage was generally greater than 30% (Figures 1 and 2) during periods of low and stable flows throughout January and February 2012 and November 2012 to January 2013. *Phormidium* coverage greater than 40% was observed in some views, although mean coverage never exceeded 14% (Figure 2). Nutrient concentrations were relatively stable across the sampling period, with a general increase in DIN during winter months, which corresponded with high and less stable flows (Figure 2). Toxins were found in 81% of mat samples (Table 1, Figure 2). Between 10 February 2012 and 17 February 2012 there was a dramatic increase in toxin concentrations from 1.2 to 82.4 mg/kg freeze-dried weight (FDW; Figure 2).

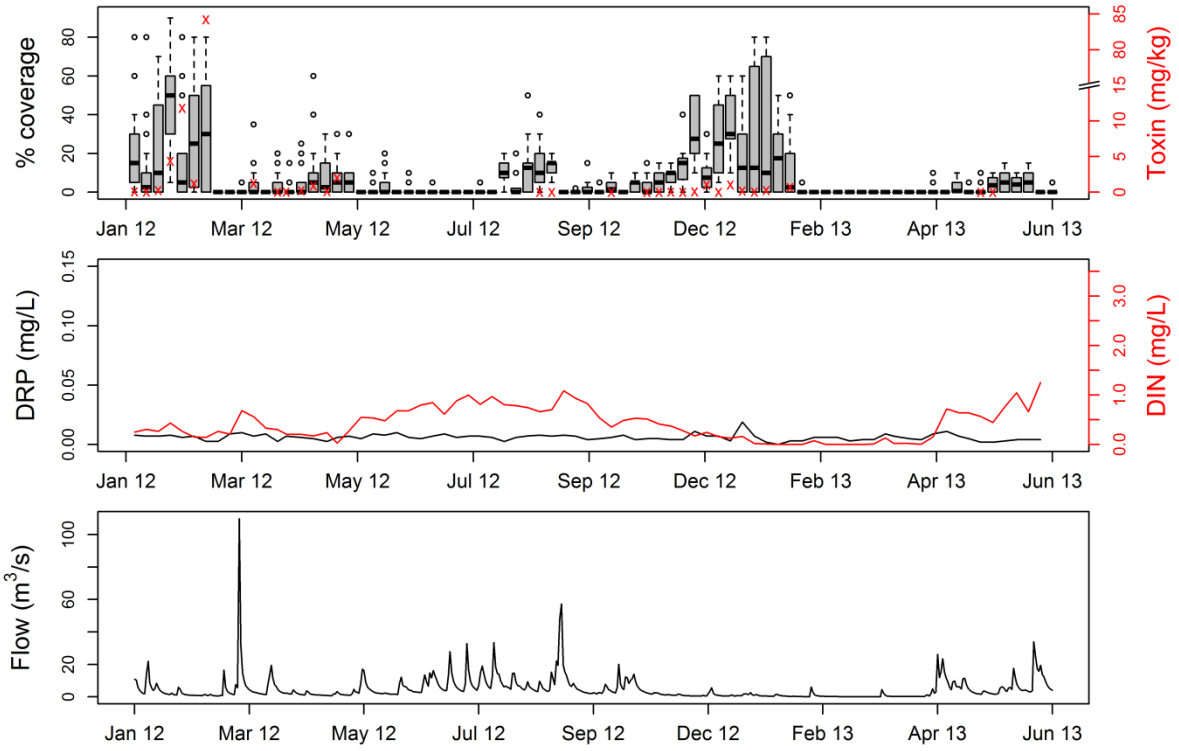

Figure 2. Makakahi River at Hamua. Top panel = *Phormidium* percentage cover and total anatoxin concentration. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the inter-quartile range if there is data that far from it. Open circles are outlines beyond this range. Middle panel = dissolved reactive phosphorus (DRP) and dissolved inorganic nitrogen (= nitrite + nitrate + ammonium; DIN). Bottom panel = daily mean river flow.

3.1.2. Manawatu River upstream of Palmerston North City Council sewage treatment plant

This site experienced a series of large flushes greater than seven times median flow (Figure 3) from July to October 2012. Both DIN and DRP were elevated during these events. Median *Phormidium* coverage was always less than 2% despite some extended stable flow periods from November 2012 to April 2013. No toxin was detected in the two samples collected for analysis at this site (Table 1, Figure 3).

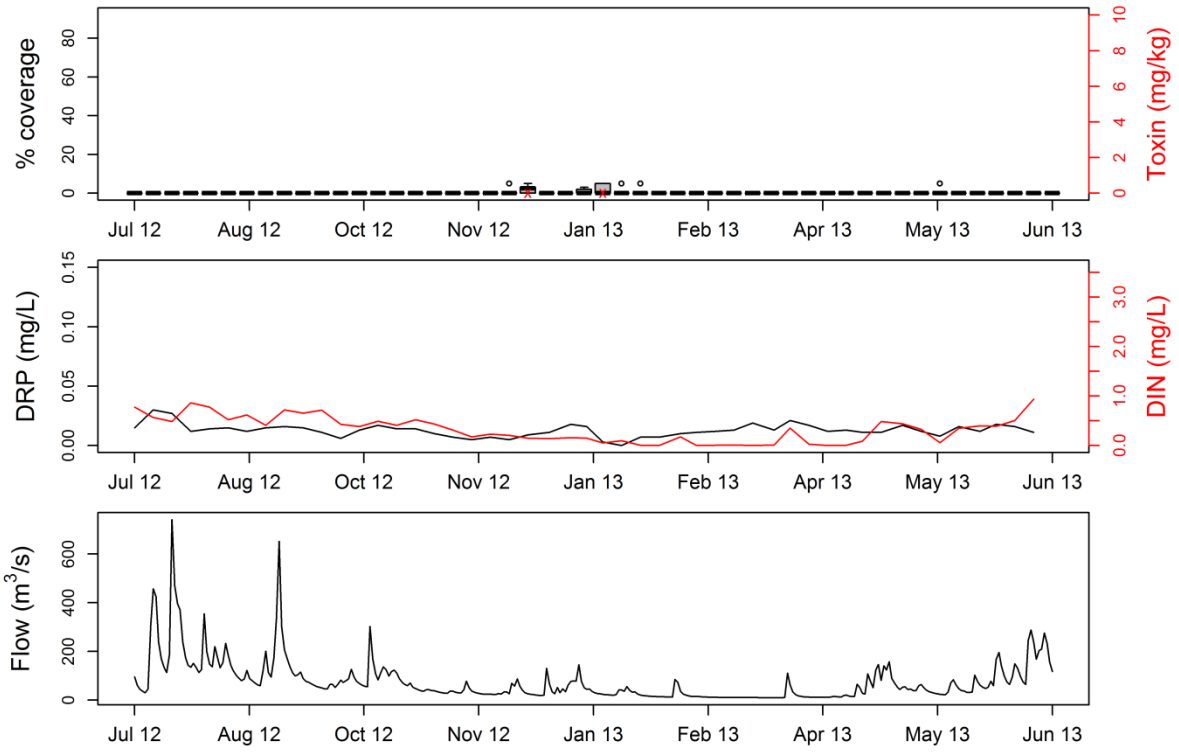

Figure 3. Manawatu River upstream of Palmerston North City Council sewage treatment plant. Top panel = *Phormidium* percentage cover and total anatoxin concentration. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the interquartile range if there is data that far from it. Open circles are outlines beyond this range. Middle panel = dissolved reactive phosphorus (DRP) and dissolved inorganic nitrogen (= nitrite + nitrate + ammonium; DIN). Bottom panel = daily mean river flow.

3.1.3. Manawatu River downstream Palmerston North City Council sewage treatment plant

Median *Phormidium* coverage was generally greater than 30% (Figure 1 and Figure 4) when flow was stable during November 2012 to May 2013. Nine of the 20 mat samples collected contained low concentrations of toxins (less than 0.2 mg/kg FDW; Table 1, Figure 4). Dissolved inorganic nitrogen and DRP followed a similar trend to the upstream sites with peaks generally occurring following large flushing events (Figure 4).

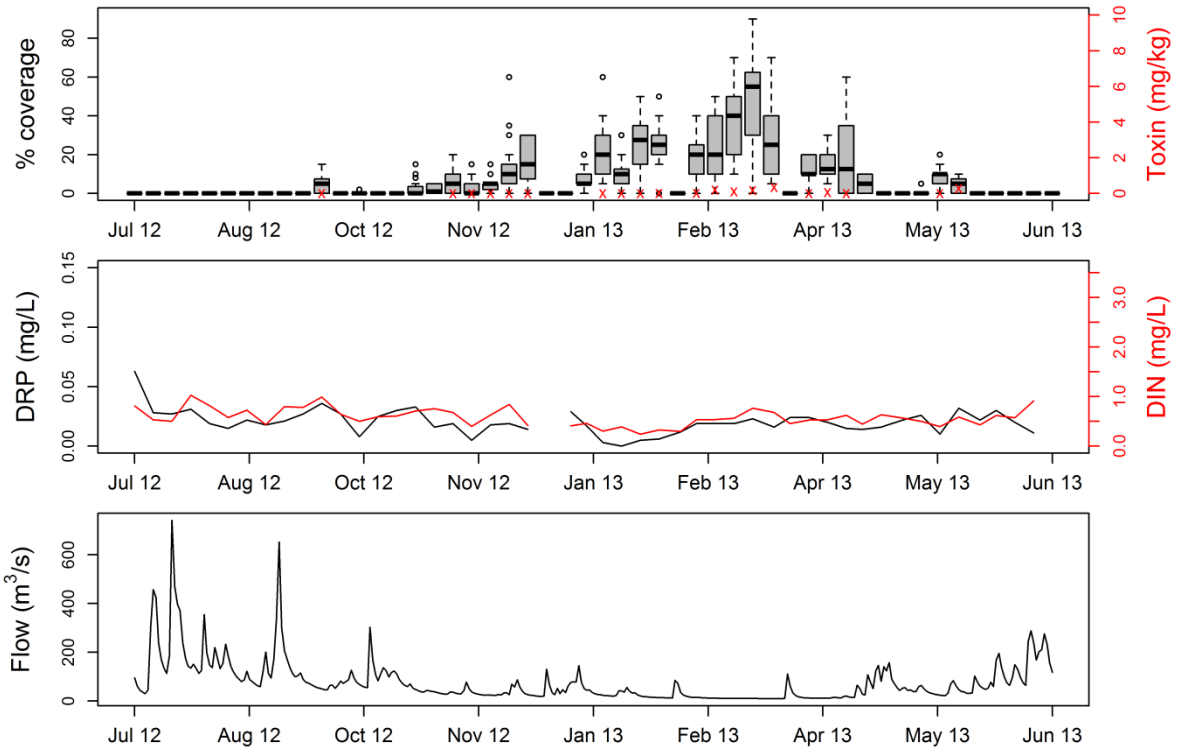

Figure 4. Manawatu River downstream of Palmerston North City Council sewage treatment plant. Top panel = *Phormidium* percentage cover and total anatoxin concentration. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the interquartile range if there is data that far from it. Open circles are outlines beyond this range. Middle panel = dissolved reactive phosphorus (DRP) and dissolved inorganic nitrogen (= nitrite + nitrate + ammonium; DIN). Bottom panel = daily mean river flow.

3.1.1. Mangatainoka River at Putara

Very low *Phormidium* coverage (max. 10% in one view) was measured at this site, despite several extended periods of relatively stable river flows (Figure 5). No samples were collected for toxin analysis (Table 1). Dissolved inorganic nitrogen and DRP were consistently low throughout the sampling period (Figure 5).

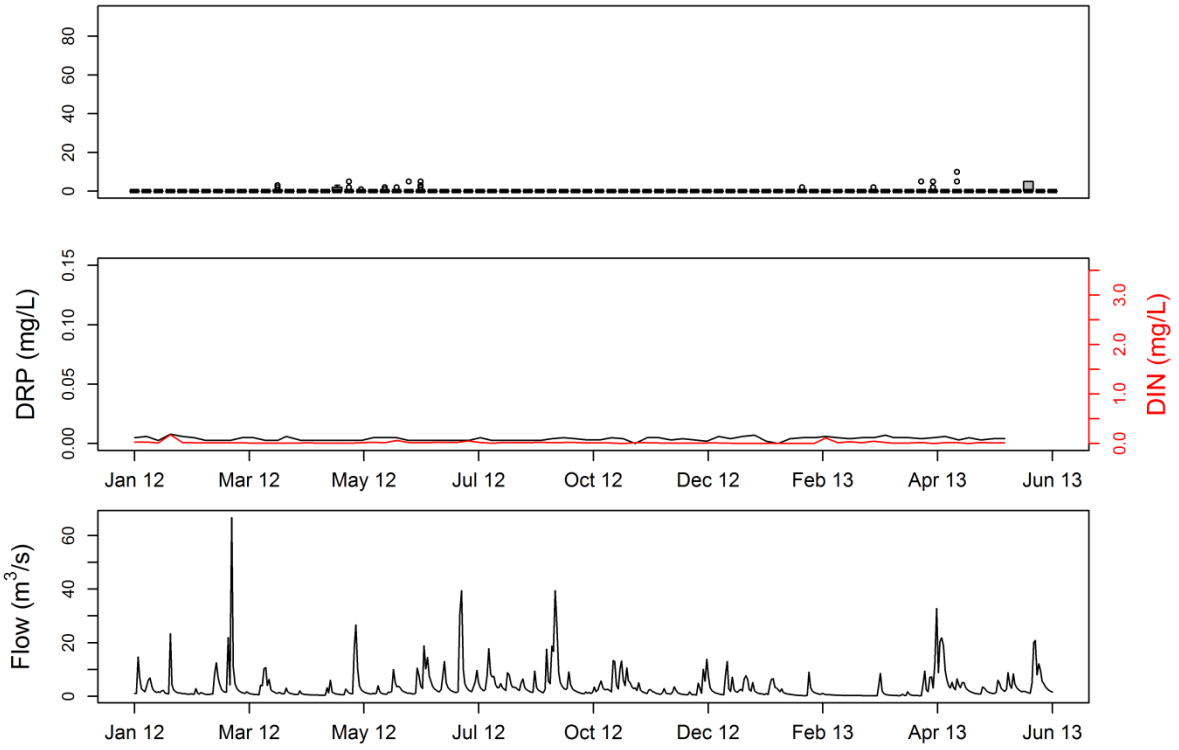

Figure 5. Mangatainoka River at Putara. Top panel = *Phormidium* percentage cover and total anatoxin concentration. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the interquartile range if there is data that far from it. Open circles are outlines beyond this range. Middle panel = dissolved reactive phosphorus (DRP) and dissolved inorganic nitrogen (= nitrite + nitrate + ammonium; DIN). Bottom panel = daily mean river flow.

3.1.2. Mangatainoka River at State Highway Two

Median *Phormidium* coverage range was from 20% to 50% during a period of low and stable flows from 6 January to 17 February 2012. Some views at this site contained 90% coverage (Figure 6). *Phormidium* coverage gradually increased when there was a period of relatively stable flows during April 2012 and January 2013 (Figure 6), and was low during February to April 2013, despite a prolonged period of stable flow. Dissolved inorganic nitrogen was generally below 0.1 mg/L during this period of stable flow, but increased after elevated river flow. Dissolved reactive phosphorus remained stable and low (less than 0.01 mg/L) throughout the sampling period. The highest number of samples (47) was collected at this site with 83% of them containing toxins (Table 1, Figure 6). The highest total concentration of toxin (16.6 mg/kg FDW) was recorded on 17 February 2012. Thereafter, toxin levels decreased and remained low until January / February 2013 when there was a short-term rise in concentrations (max.16.6 mg/kg FDW; Figure 6).

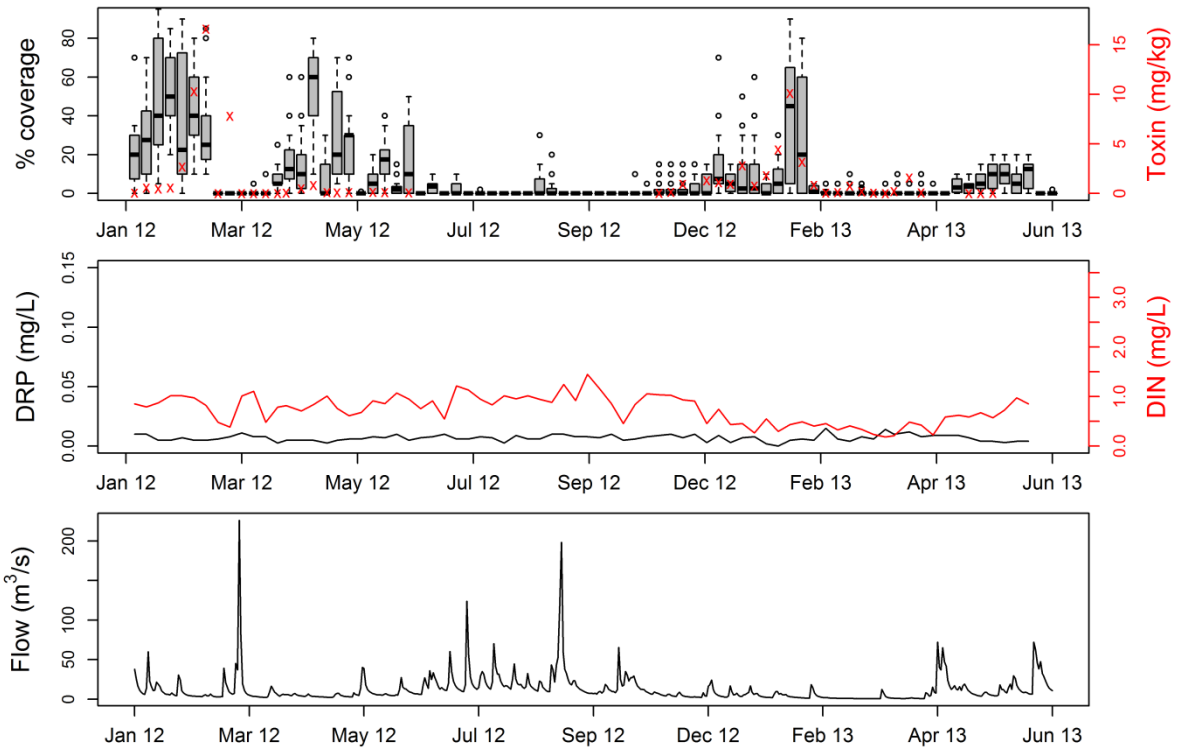

Figure 6. Mangatainoka River at State Highway Two. Top panel = *Phormidium* percentage cover and total anatoxin concentration. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the interquartile range if there is data that far from it. Open circles are outlines beyond this range. Middle panel = dissolved reactive phosphorus (DRP) and dissolved inorganic nitrogen (= nitrite + nitrate + ammonium; DIN). Bottom panel = daily mean river flow.

3.1.3. Oroua River upstream of Feilding sewage treatment plant

Phormidium coverage remained low (median less than 5%) at this site despite several extended and relatively stable periods of river flow throughout the study. High coverage (60%) was recorded in one view on one occasion (Table 1, Figure 7). No toxins were detected in the eight samples collected for analysis at this site (Table 1, Figure 7). Dissolved inorganic nitrogen and DRP were highly variable across the study period. These fluctuations did not necessarily align with changes in flow, although in general, DIN and DRP were higher in the winter months.

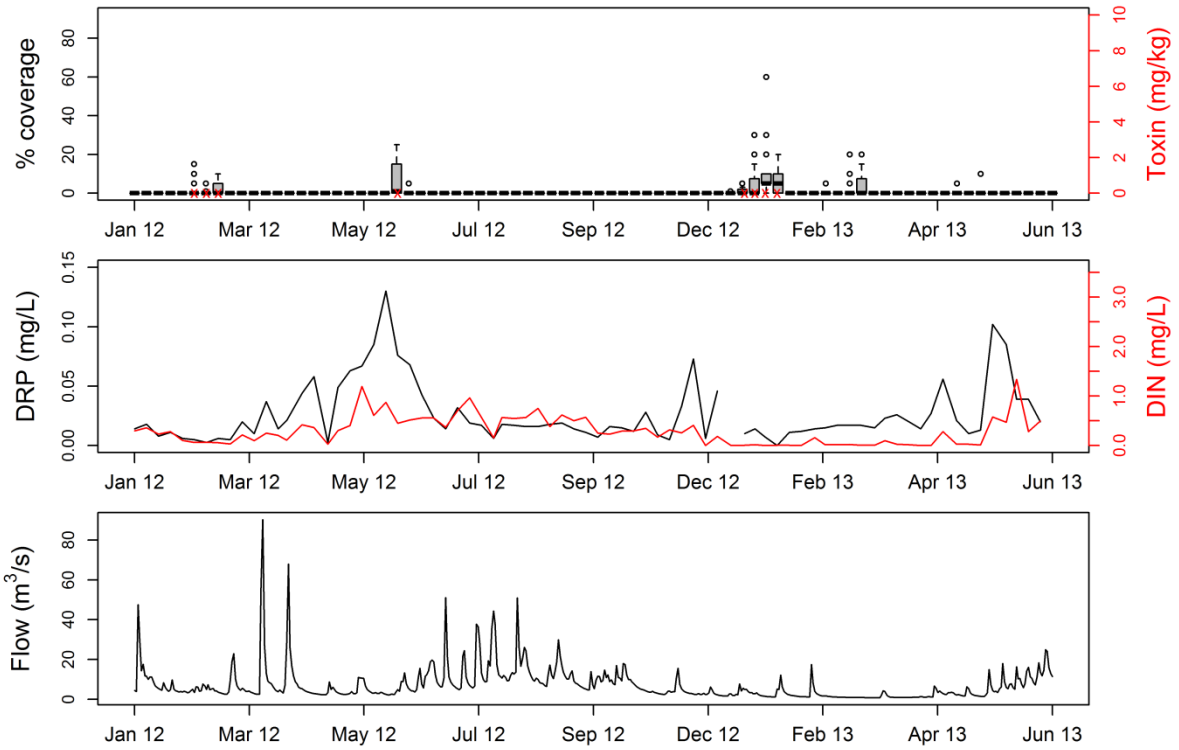

Figure 7. Oroua River upstream of Feilding sewage treatment plant. Top panel = *Phormidium* percentage cover and total anatoxin concentration. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the interquartile range if there is data that far from it. Open circles are outlines beyond this range. Middle panel = dissolved reactive phosphorus (DRP) and dissolved inorganic nitrogen (= nitrite + nitrate + ammonium; DIN). Bottom panel = daily mean river flow.

3.1.4. Oroua River downstream of Feilding sewage treatment plant

This site followed a similar trend to the upstream Oroua River site from January to May 2012. *Phormidium* coverage was only recorded on five occasions and the median never exceeding 5% (Figure 8). In contrast, median *Phormidium* coverage was greater than 30% on several occasions in January 2013 (Figure 8). No toxins were detected in any of the 11 samples collected (Table 1, Figure 8). Dissolved inorganic nitrogen and DRP were highly variable across the study period, and these fluctuations did not necessarily align with changes in flow (Figure 8).

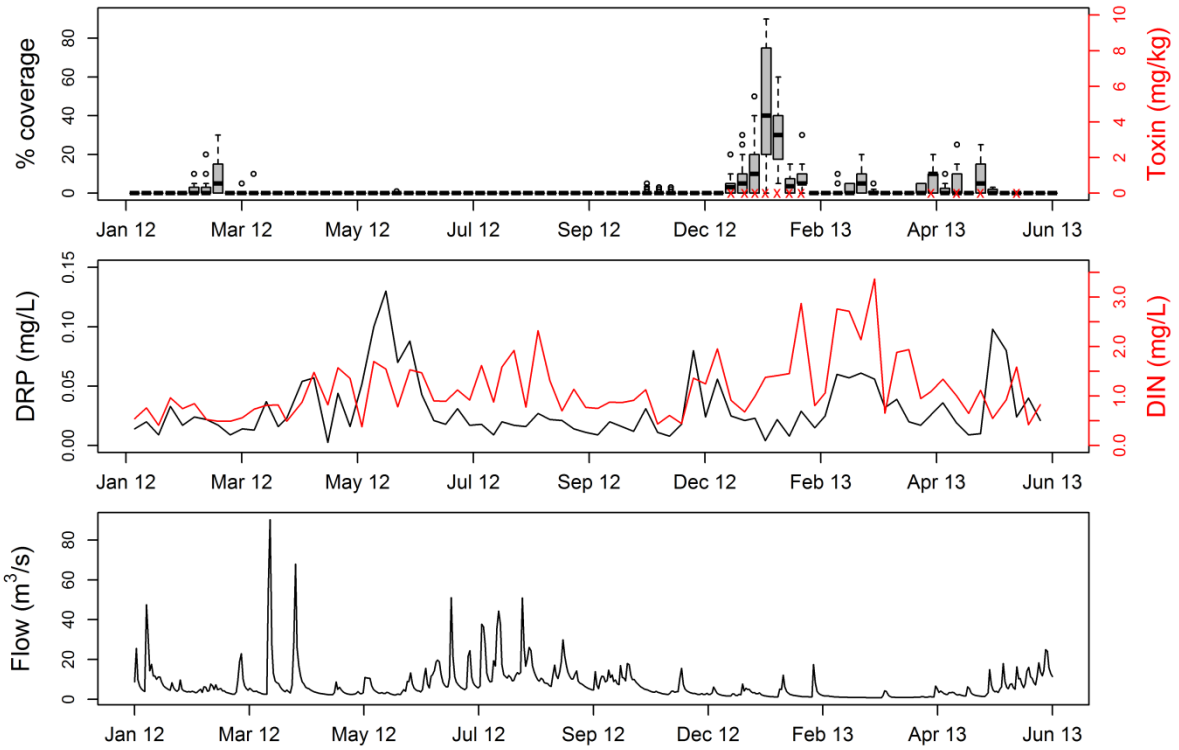

Figure 8. Oroua River downstream of Feilding sewage treatment plant. Top panel = *Phormidium* percentage cover and total anatoxin concentration. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the interquartile range if there is data that far from it. Open circles are outlines beyond this range. Middle panel = dissolved reactive phosphorus (DRP) and dissolved inorganic nitrogen (= nitrite + nitrate + ammonium; DIN). Bottom panel = daily mean river flow.

3.1.5. Oruakeretaki River at State Highway Two

Phormidium mats were detected, but never exceeded median coverage of 15% at this site during the relatively stable periods of river flow in January to April 2013 (Figure 9). Fifteen of the 16 mats collected contained toxins; however, concentrations were low (less than 0.9 mg/kg FDW, Table 1, Figure 9). Dissolved reactive phosphorus concentration was relatively stable throughout the study period. In contrast DIN was more variable and was generally higher during the winter months. There was a pronounced peak in DIN (3.6 mg/L) associated with higher river flows on 17 May 2013.

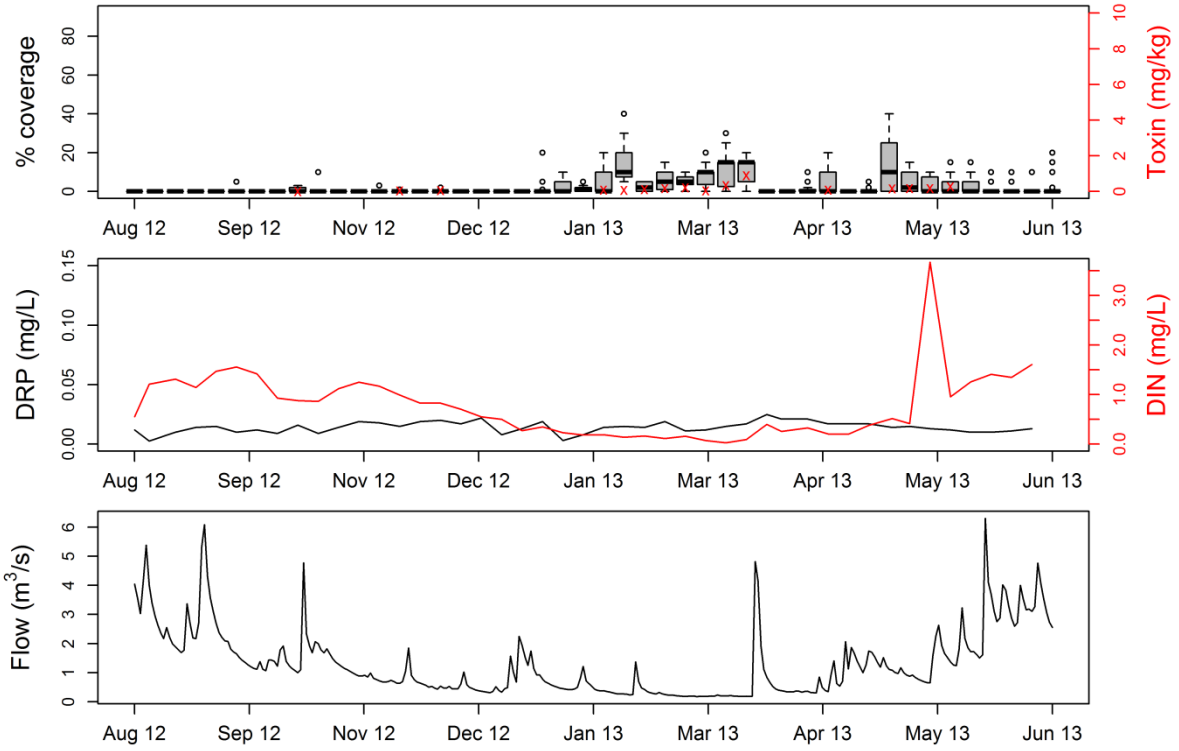

Figure 9. Oruakeretaki River at State Highway Two. Top panel = *Phormidium* percentage cover and total anatoxin concentration. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the interquartile range if there is data that far from it. Open circles are outlines beyond this range. Middle panel = dissolved reactive phosphorus (DRP) and dissolved inorganic nitrogen (= nitrite + nitrate + ammonium; DIN). Bottom panel = daily mean river flow.

3.1.6. Tiraumea River at Ngaturi

Phormidium mats were detected at this site during relatively stable periods of river flow in February 2012, April 2012 and December 2012 to April 2013. However, the coverage never exceeded a median of 20% (Figure 10). Only six of the 19 samples tested positive for anatoxins, and concentrations were low (max. 0.2 mg/kg FDW; Table 1, Figure 10). Dissolved inorganic nitrogen and DRP were relatively stable throughout the sampling period and observed fluctuations did not necessarily align with changes in flow (Figure 10).

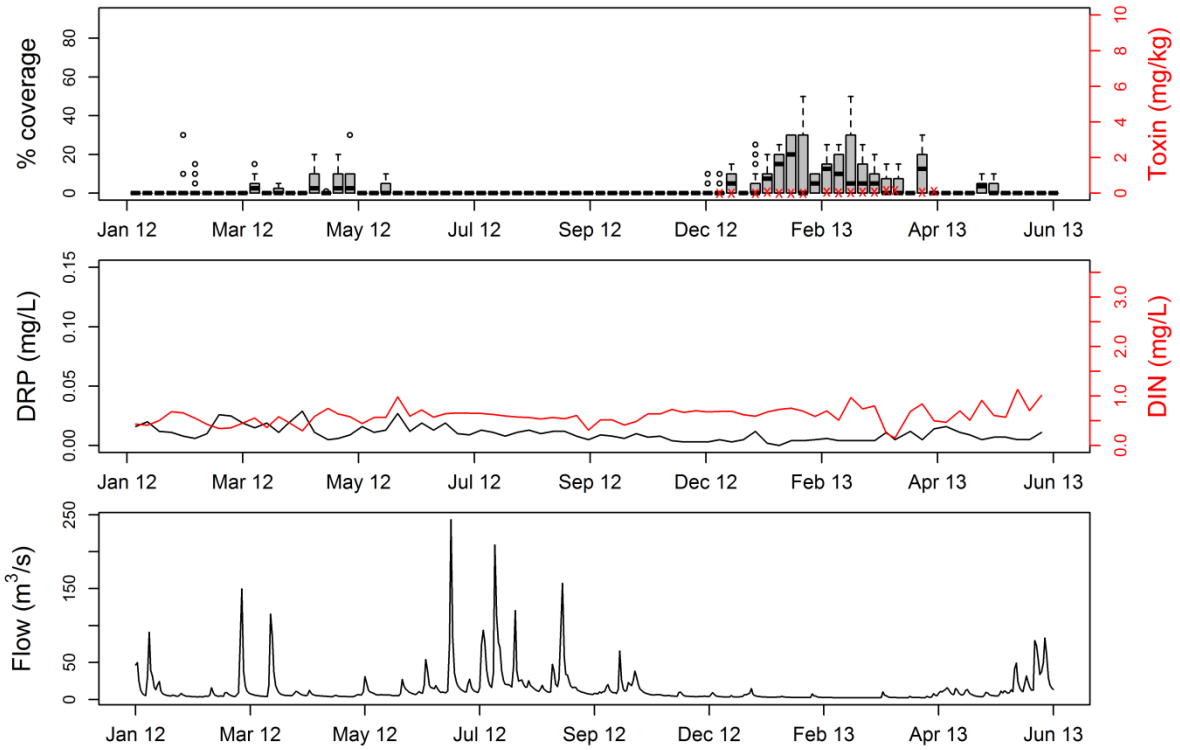

Figure 10. Tiraumea River at Ngaturi. Top panel = *Phormidium* percentage cover and total anatoxin concentration. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the interquartile range if there is data that far from it. Open circles are outlines beyond this range. Middle panel = dissolved reactive phosphorus (DRP) and dissolved inorganic nitrogen (= nitrite + nitrate + ammonium; DIN). Bottom panel = daily mean river flow.

3.1.7. Tokomaru River at Horseshoe Bend

The highest median *Phormidium* coverage of the study (80%) was recorded at this site (11 May 2012; Figure 11). Ninety percent of the 29 samples collected contained moderate concentrations (ca. 5 mg/kg to 6 mg/kg FDW) of toxins (Table 1, Figure 11) and this was reflected in measurements on several occasions. This site experienced numerous flushing events throughout the sampling period (Figure 11). Flushes that were greater than three times the long-term median flow did not appear to remove *Phormidium* mats. Dissolved inorganic nitrogen and DRP were consistently low throughout the study period (Figure 11).

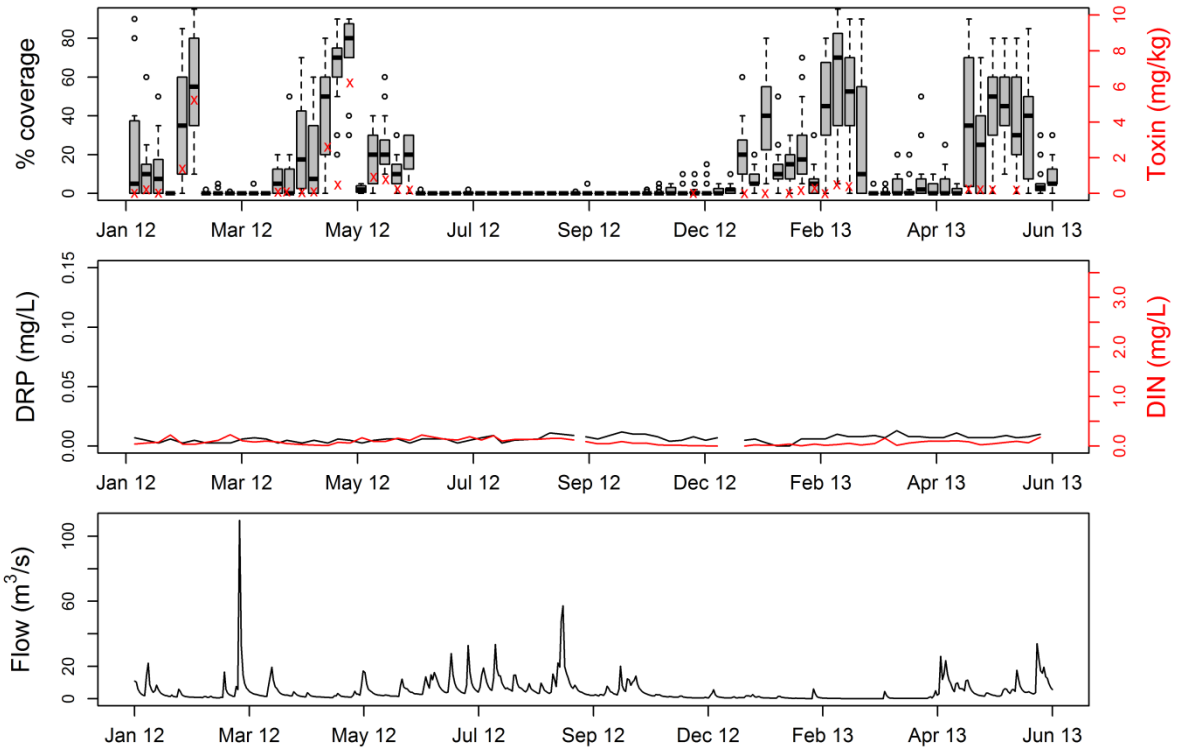

Figure 11. Tokomaru River at Horseshoe Bend. Top panel = *Phormidium* percentage cover and total anatoxin concentration. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the interquartile range if there is data that far from it. Open circles are outlines beyond this range. Middle panel = dissolved reactive phosphorus (DRP) and dissolved inorganic nitrogen (= nitrite + nitrate + ammonium; DIN). Bottom panel = daily mean river flow.

3.2. River flow and *Phormidium* coverage

Quantile regressions identified the 90th percentile as most appropriate for determining the flushing flow[1] at most sites. The exception was at Tokomaru River at Horseshoe Bend where the 85th percentile provided the best fit to the data. The flushing flow varied markedly between sites (Figures 12 and 13, Table 2). The Manawatu River downstream of STP site required the lowest flushing flow (0.65 'times median'). In contrast, the Mangatainoka River at SH2 required a flushing flow event 5.36 times the median flow to reduce *Phormidium* cover below 20% (Figure 12). There was insufficient data to use this approach at the Oroua River downstream of Feilding STP because the mean *Phormidium* coverage was greater than 20% on only two sampling occasions (Figure 13).

Table 2. The 'times median flow' *i.e.* the amount above the river flow is above the long-term median, and actual river flows at which 80%, 85% and 90% of records are below 20% *Phormidium* coverage (derived from Figures 12–14). Shading indicates the values that are recommended for management purposes. D/S = downstream, STP = sewage treatment plant. SH2 = State Highway Two. * insufficient data for this approach to be used.

	Times median			River flow (m^3/s)		
Percentile	**80**	**85**	**90**	**80**	**85**	**90**
Makakahi River at Hamua	0.97	1.39	2.54	3.08	4.42	8.08
Manawatu River D/S STP	0.38	0.51	0.65	1.21	1.62	2.07
Mangatainoka River at SH2	0.72	1.46	5.36	2.29	4.64	17.04
Tokomaru River at Horseshoe Bend	6.75	16.53	248.96	21.47	52.57	791.69

[1] Defined in this report as the amount the river flow was required to be above the long-term median, at which 90% (or 85%) of records were below 20% *Phormidium* coverage.

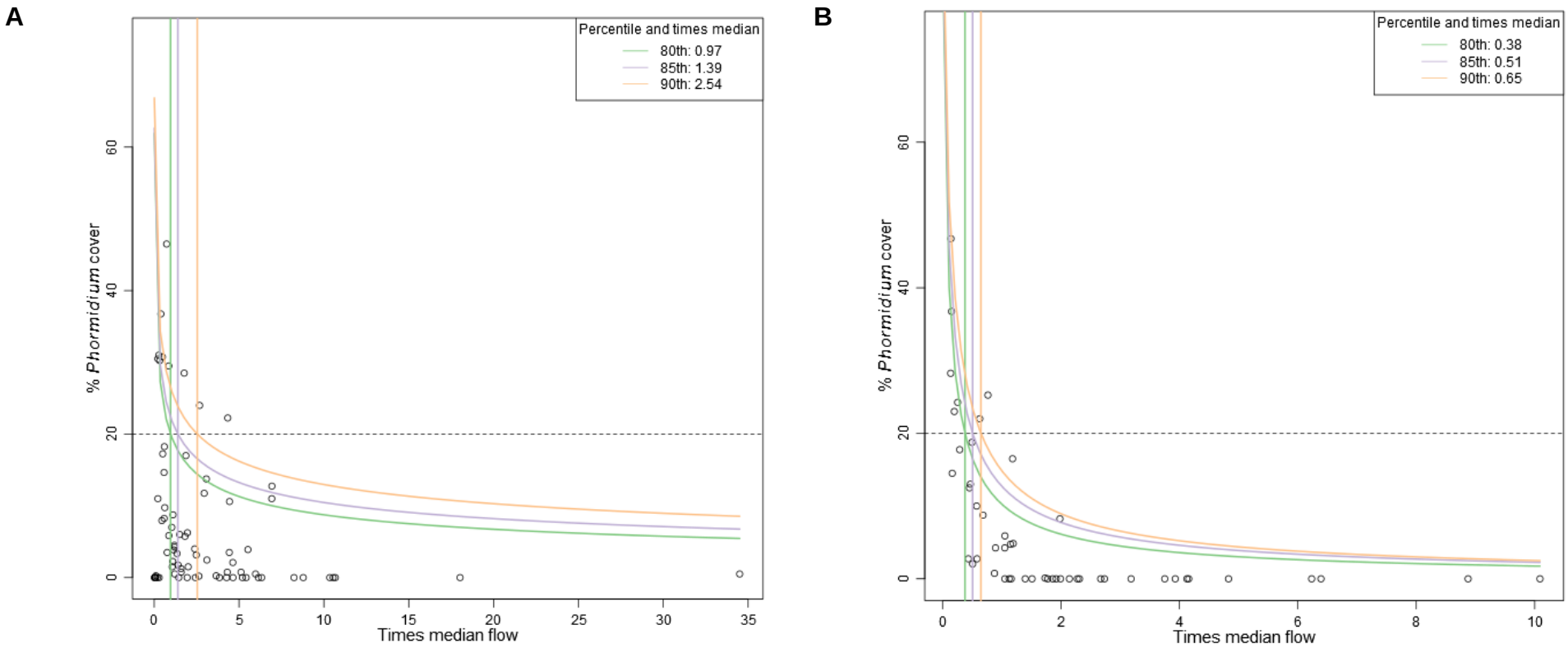

Figure 12. Mean *Phormidium* mat cover and magnitude of maximum daily flow above long-term median river flow on the day of sampling or during the six days prior derived by 0.80, 0.85 or 0.90 quantile regression for; A) Makakahi River at Hamua and B) Manawatu River downstream of sewerage treatment plant.

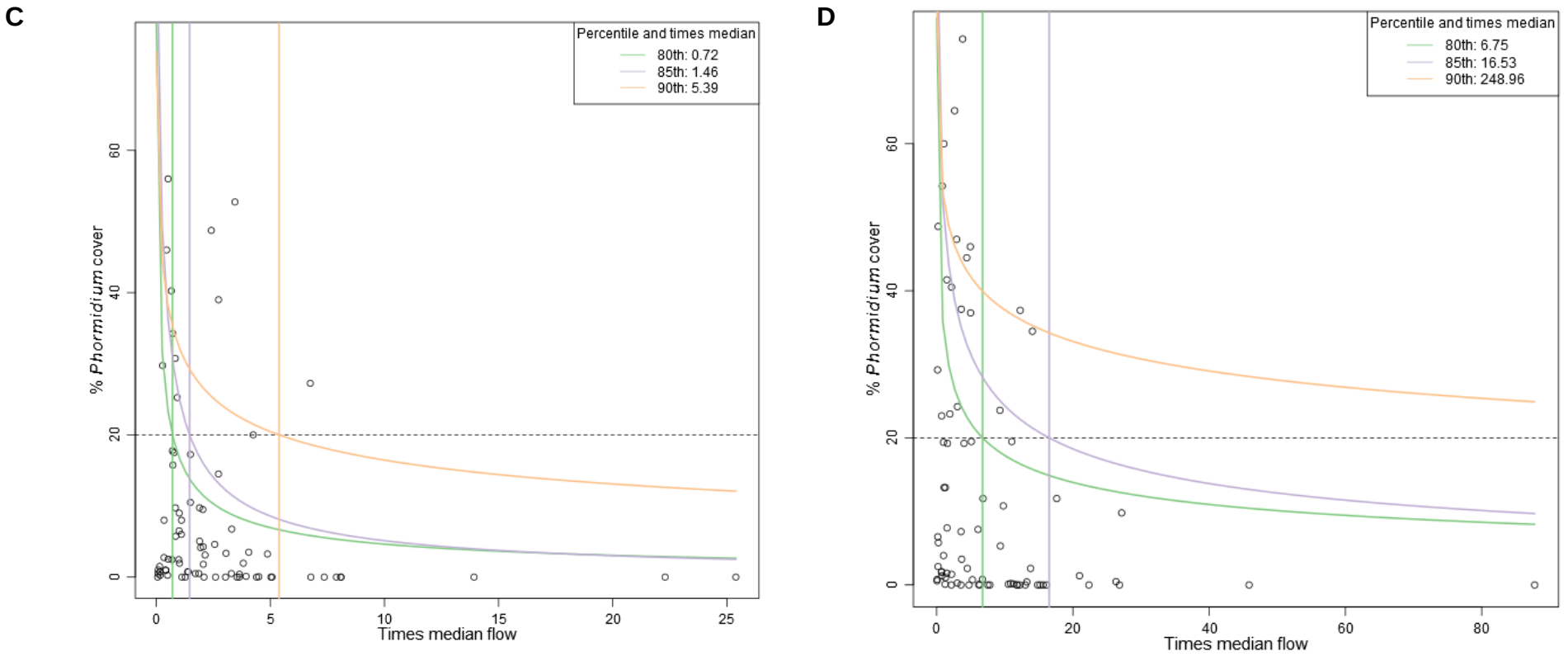

Figure 13. Mean *Phormidium* mat cover and magnitude of maximum daily flow above long-term median river flow on the day of sampling or during the six days prior derived by 0.80, 0.85 or 0.90 quantile regression for; A) Mangatainoka River at State Highway Two, and B) Tokomaru River at Horseshoe Bend.

3.3. Nutrients

There were marked differences in nutrient concentrations at the three sites with almost no *Phormidium*. Both DRP and DIN were low (less than 0.01 and 0.04 mg/L; Figure 15C) at the Mangatainoka River at Putara site. In contrast, DRP was more than 0.01 mg/L and DIN was also elevated (greater than 0.18 and 0.1 mg/L respectively; Figures 14B and 15A) at the Manawatu River upstream of the PNCC STP and Oroua River upstream of Feilding STP sites.

The mean coverage of *Phormidium* at both the Oruakeretaki River at SH2 and Tiraumea River at Ngaturi sites was always less than 20%. Dissolved reactive phosphorus was generally more than 0.1 mg/L and DIN was usually more than 0.5 mg/L at the Oruakeretaki River at SH2 site (Figure 16 B). Dissolved reactive phosphorus was usually always greater than 0.1 mg/L and DIN was usually greater than 0.5 mg/L at the Tiraumea River at Ngaturi site. Mean *Phormidium* coverage only exceeded 10% when DRP was less than 0.1 mg/L (Figure 16 C).

The five sites with *Phormidium* blooms can be grouped based on their DRP and DIN concentrations (Figure 17).

1. Low DRP (less than 0.01 mg/L) and elevated DIN (greater than 0.1 mg/L). This includes the Makakahi River at Hamua, and Mangatainoka River at SH2 sites.
2. Sites downstream of STPs with both elevated DIN and DRP (greater than 0.1 mg/L and 0.01 mg/L respectively). This includes the Manawatu River downstream of the PNCC STP and Oroua River downstream Feilding STP sites.
3. Very low DRP and DIN (less than 0.01 mg/L and 0.06 mg/L, respectively). The only site in this category was Tokomaru River at Horseshoe Bend.

The analysis of the 45 sites from the Manawatu region also identified the same three groups (Figure 17). The sites with high *Phormidium* coverage generally had low DRP (ca. 0.01 mg/L), except the sites below STPs when DRP was higher (ca. 0.02 mg/L). There were two notable exceptions; site 31—Oroua River at Awahuri Bridge and site 5—Mangawhero River at Pakihi Road Bridge. There are sewage treatment plants upstream of both of these sites. While these sites are not directly downstream of a STP, it is likely that they are impacted.

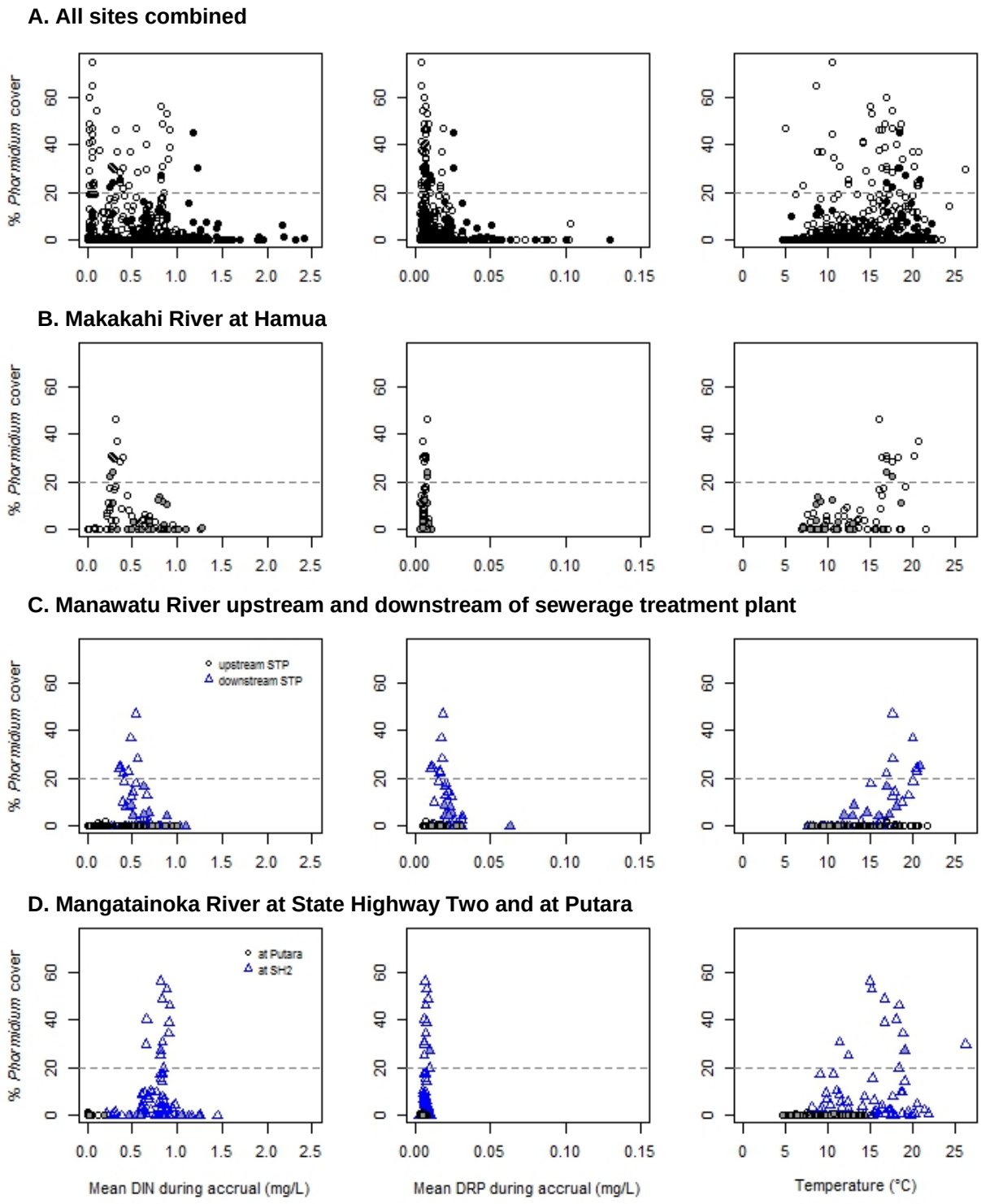

Figure 14. Relationship between mean *Phormidium* coverage and dissolved reactive phosphorus (DRP), dissolved inorganic nitrogen (DIN), and temperature. Dissolved inorganic nitrogen and DRP are a cumulative mean over the accrual period. For; A) enclosed circles are those which are flow affected. This is defined as a flow greater than the 90th (or 85th for Tokomaru River) percentile (see Table 2).

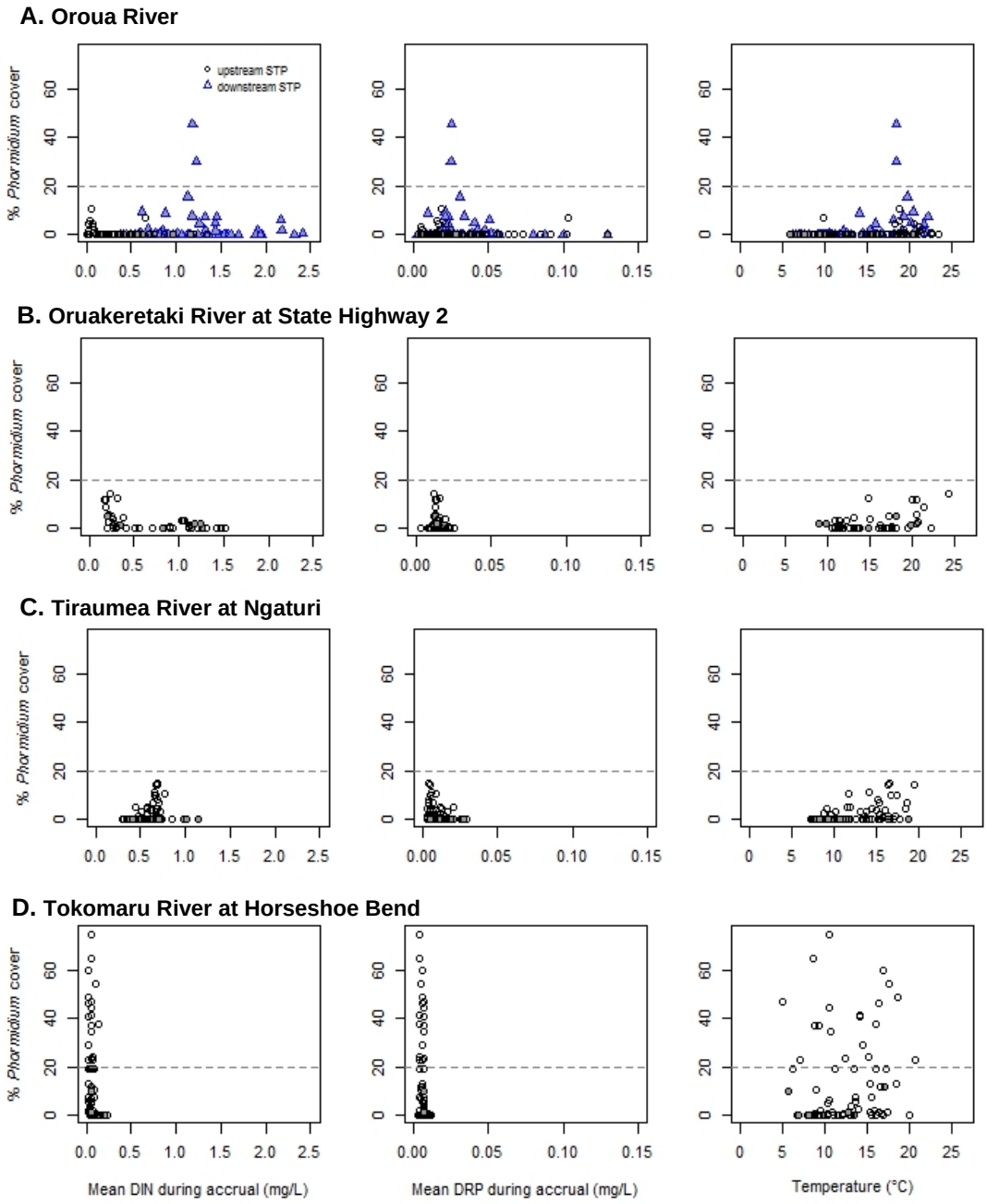

Figure 15. Relationship between mean *Phormidium* coverage and dissolved reactive phosphorus (DRP), dissolved inorganic nitrogen (DIN), and temperature. Dissolved inorganic nitrogen and DRP are a cumulative mean over the accrual period.

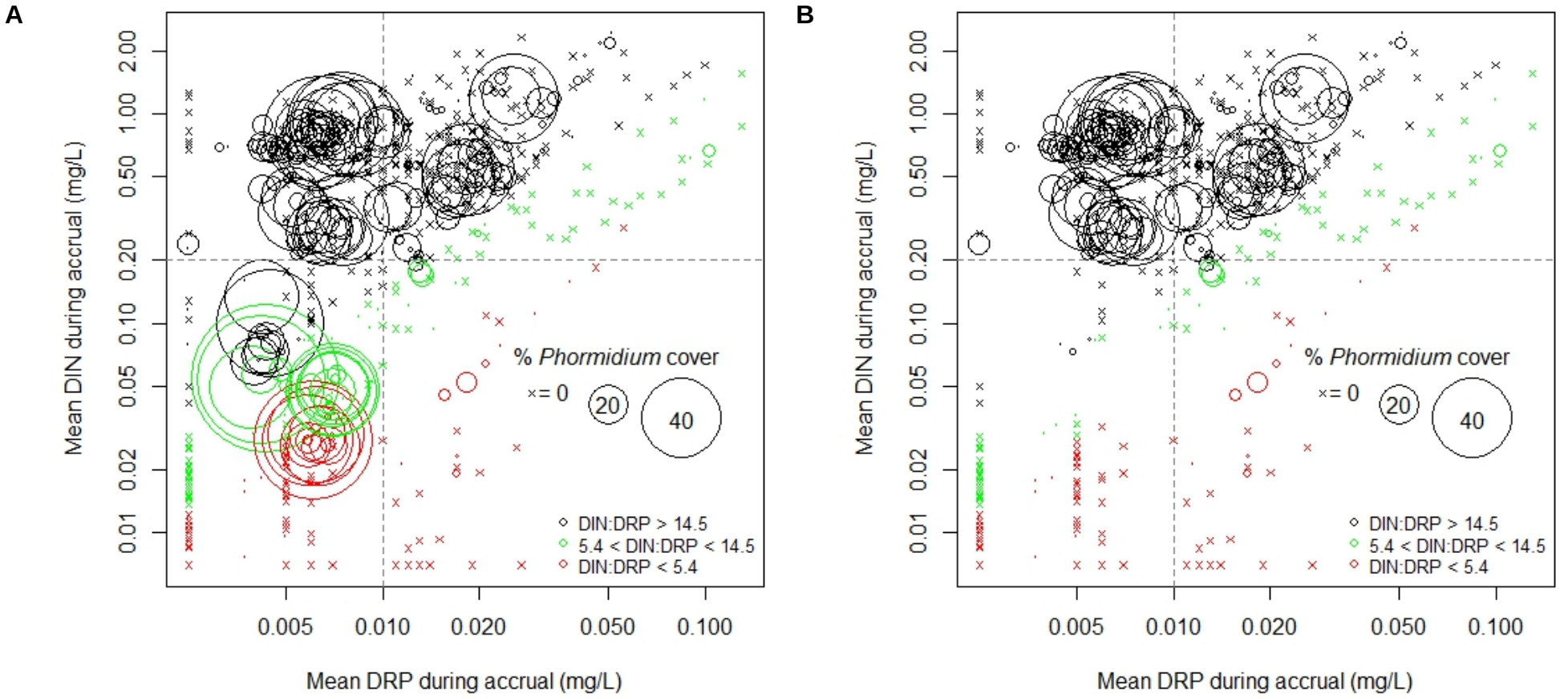

Figure 16. Relationship between mean *Phormidium* coverage and dissolved reactive phosphorus (DRP) and dissolved inorganic nitrogen (DIN). Dissolved inorganic nitrogen and DRP are a cumulative mean over the accrual period. Colours indicate potential nutrient limitation as given Kahlert 1998; > 14.5 = phosphorus limited and < 5.4 suggest nitrogen limitation. A) all sampling points, B) all sampling points except Tokomaru River at Horseshoe Bend. Dashed lines show suggested DRP and DIN thresholds that favour *Phormidium* blooms. All points in the top right corner with *Phormidium* coverage are downstream of sewerage treatment plants.

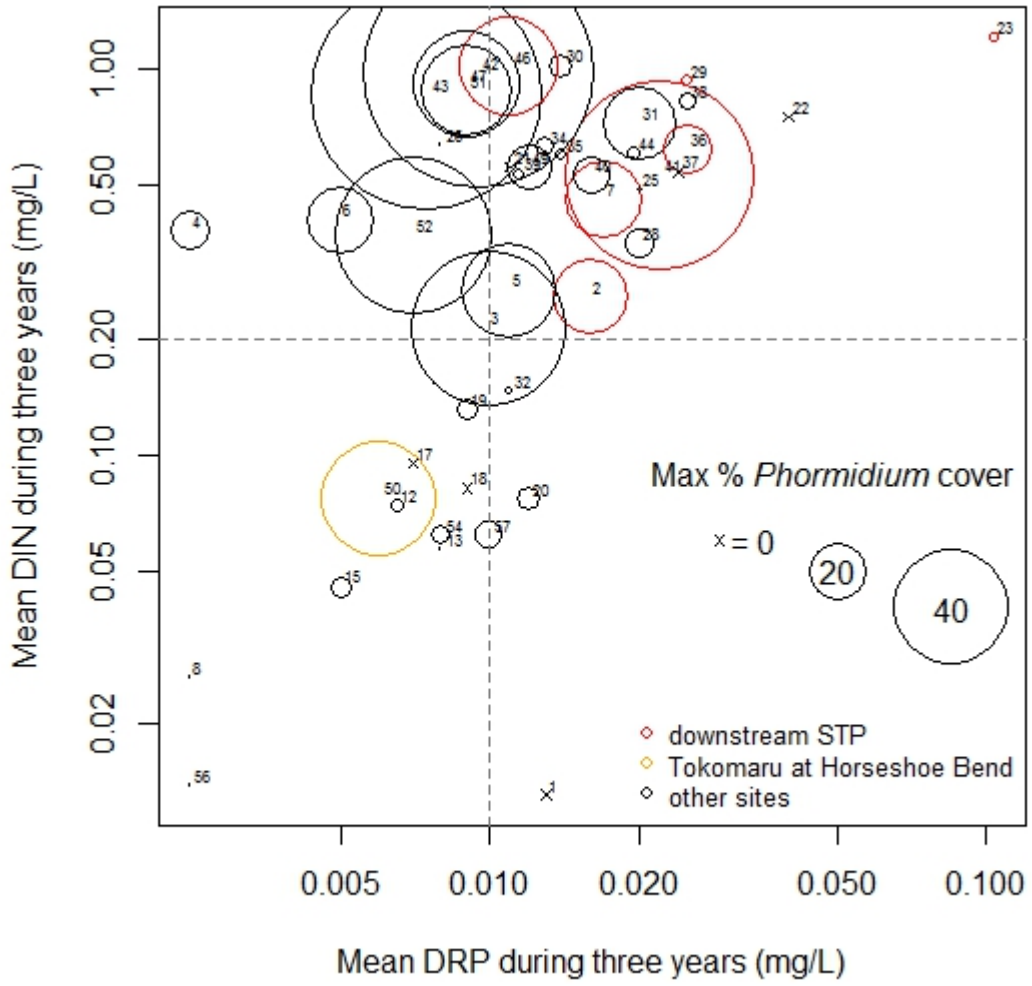

Figure 17. Relationship between maximum *Phormidium* coverage and mean dissolved reactive phosphorus (DRP) and dissolved inorganic nitrogen (DIN) at 45 sites over a 2-year period (2011–2012) in the Manawatu region (see Appendix 2 for a list of sites related to numbers in graph). Dashed lines show suggested DRP and DIN thresholds that favour *Phormidium* blooms.

3.4. Water temperature and *Phormidium* coverage

Phormidium mats were present in a wide range of water temperatures (4.9˚C to 26.2˚C). Although blooms (> 20% coverage) tended to occur in water temperature above 15˚C (Figures 14 and 15), there was no correlation ($F_{1, 179} = 0.41$, $r^2 = 0.002$, $p = 0.52$) between mean percentage coverage of *Phormidium* mats and water temperature.

3.5. Substrate

The dominant substrate at all sampling sites were large (12 cm–25 cm) and small (6 cm–12 cm) cobbles. These accounted for greater than 42% of the substrate (Figure 18). The substrate at the Mangatainoka River at Putara and Tokomaru River at Horseshoe Bend sites contained a markedly greater proportion of boulders (26% and 21%) compared to other sites (Figure 18).

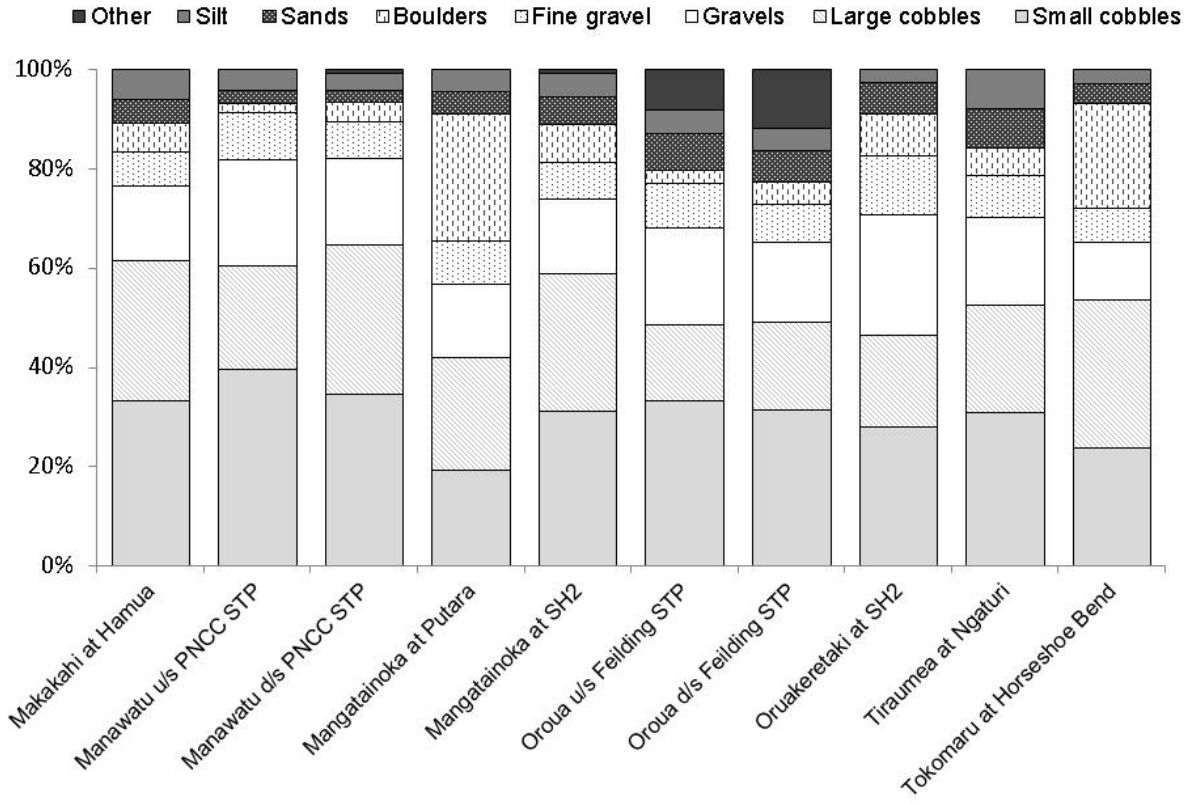

Figure 18. Mean percentage coverage of different substrate size classes over surveyed reaches across the entire study period at each sampling site. 'Other' referring to macrophytes, moss or bedrock u/s = upstream, d/s = downstream, PNCC = Palmerston North City Council, STP = sewage treatment plant. SH2 = State Highway Two.

3.6. Anatoxins

To simplify visualisation of the toxin data, the concentrations of ATX, HTX, dhATX and dhHTX in each sample were combined and a total toxin concentration used for Figures 2 to 11 and Figure 19. Full toxin results showing each variant are given in Appendix 3.

Anatoxins were detected in variable concentrations in the samples from the 10 sites. There were temporal variations in the presence of toxins within and among sites (Figures 2 to 11). There were no sites where all samples collected contained toxins (Table 1). However, there were three sites where no toxins were detected (Manawatu River upstream of the PNCC STP and the Oroua River upstream and downstream of the Feilding STP). The highest toxin concentration measured in the study was at Makakahi River at Hamua (82.38 mg/kg, 17 February 2012). This was markedly higher than the next highest concentration measured on the same date (16.6 mg/kg, Mangatainoka River at SH2).

There was no correlation between mean percentage *Phormidium* coverage and total toxin concentration ($F_{1, 179} = 5.45$, $r^2 = 0.030$, $p = 0.02$; Figure 19A). The highest toxin concentration recorded (82.4 mg/kg FDW) corresponded to a mean *Phormidium* percentage coverage of 31% (Figure 19A). When *Phormidium* percentage coverage was greatest both high (*e.g.* 74%, 6.2 mg/kg FDW, Tokomaru River at Horseshoe Bend, 11 May 2012) and low (*e.g.* 65%, 0.5 mg/kg FDW, Tokomaru River at Horseshoe Bend, 4 May 2012) toxin concentrations were measured (Figure 19A).

Toxins were detected in mats in a wide range of water temperatures (6˚C to 26˚C). There was no correlation between the concentration of total toxins and water temperature ($F_{1, 179} = 0.21$, $r^2 = 0.001$, $p = 0.65$, Figure 19A). Water temperature did not appear to influence the total amount of toxin produced. For example, within sites similar total toxin concentrations where measured at contrasting water temperatures *i.e.* 0.5 mg/kg FDW at 16.9˚C, and 0.5 mg/kg FDW at 8.6˚C (Tokomaru River at Horseshoe Bend).

There was no correlation between the concentration of total toxins and DIN ($F_{1, 179} = 0.59$, $r^2 = 0.003$, $p = 0.44$) or DRP ($F_{1, 179} = 1.78$, $r^2 = 0.01$, $p = 0.18$, Figure 19A). Within sites there was marked variability in total toxin concentrations when DIN or DRP concentrations were similar *e.g.* Mangatainoka River at SH2:

- on **9 November 2012—**DIN 1.03 mg/L, toxins 0.02 mg/kg versus **3 February 2012** (DIN 1.02 mg/L, toxins 2.7 mg/kg.
- on **12 April 2013—**DRP 0.08 mg/L, toxins 0.08 mg/kg) versus **2 March 2012** DRP 0.08 mg/L, toxins 7.8 mg/kg.

No ATX variant was detected in any of the samples (Figure 20; Appendix 2). Over the entire sampling period HTX was the dominant variant at four sites (Manawatu River downstream PNCC STP, Oruakeretaki River at SH2, Tiraumea River at Ngaturi, and Tokomaru River at Horseshoe Bend) accounting for greater than 50% of the total toxin concentration. Dihydro-homoanatoxin-a was dominant at the other two sites (Makakahi River at Hamua and Mangatainoka River at SH2; Figure 20). There was

some temporal variability in the dominant variant among sites but no obvious patterns were observed (Appendix 2).

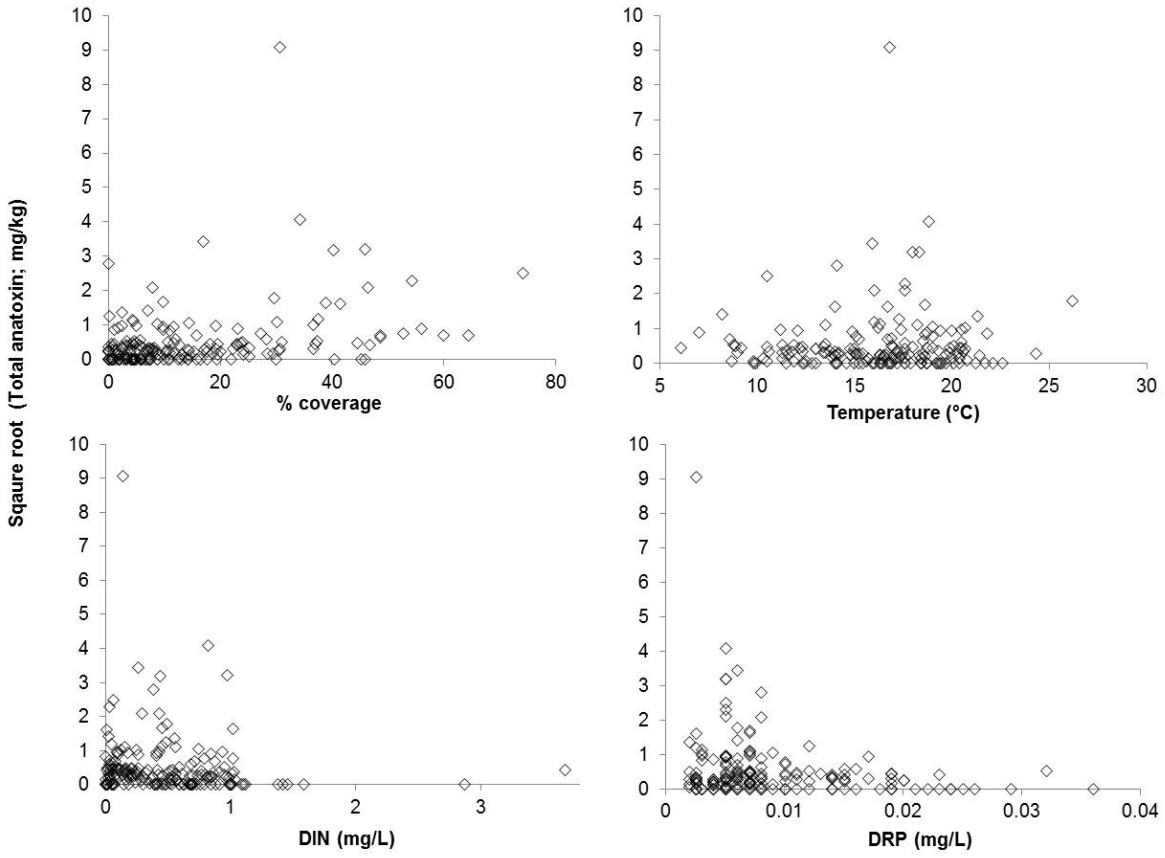

Figure 19. Total anatoxin toxin concentration (mg/kg of freeze-dried material) versus: A) mean *Phormidium* coverage, B) water temperature, C) dissolved inorganic nitrogen (DIN), and D) dissolved reactive phosphorus (DRP). For all graphs temperature and nutrient data is at time the sample was collected.

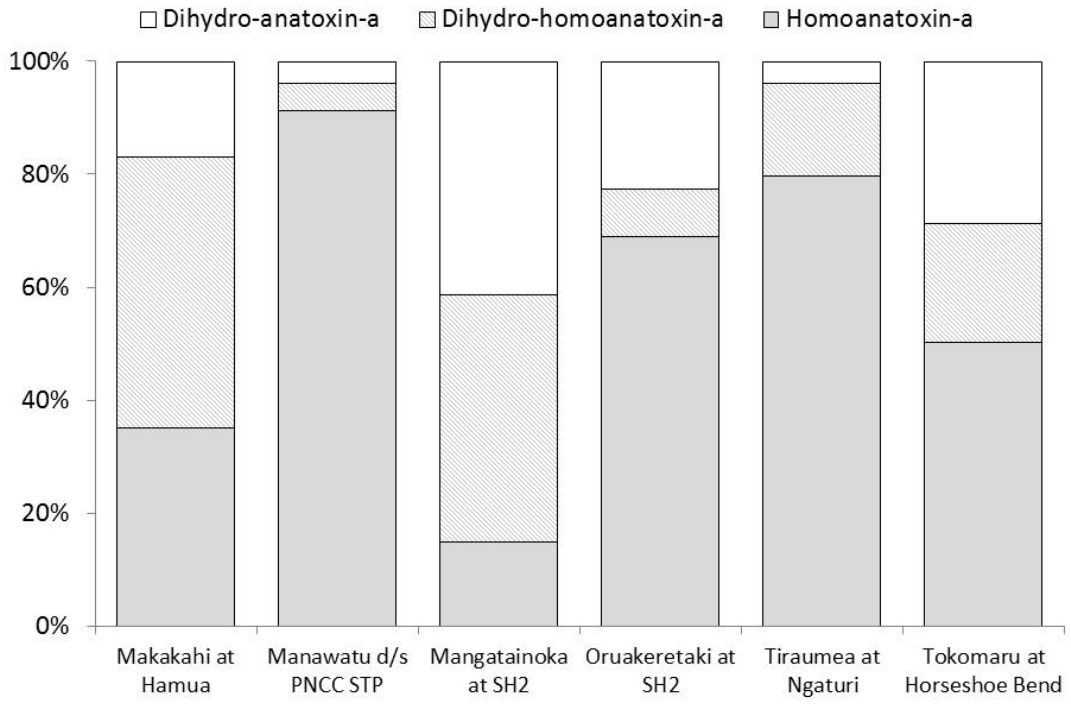

Figure 20. Staked bar graph showing the percentage of each anatoxin variant present at each site over all samples. PNCC STP = Palmerston North City Council sewage treatment plant, SH2 = State Highway Two.

4. DISCUSSION

4.1. River flow

The basic model for control of periphyton biomass identifies hydrologic disturbance as the primary control, whilst nutrients operate within this by regulating the rate of accrual during stable periods (Biggs 1995, Biggs *et al*. 2005).

Adopting the suggestion that a flushing flow three times median is sufficient to remove *Phormidium* mats (Milne & Watts 2006) does not appear to be applicable to all study sites in this investigation. The analysis in this study shows that a flushing flow, three times median, was sufficient to reduce *Phormidium* mat cover at the Manawatu River downstream of the PNCC STP, Makakahi River at Hamua, and Oroua River downstream Feilding STP sites (although further data is needed for this site). However, this does not take stage of growth or time of year into consideration. Markedly higher flows were required to reduce *Phormidium* coverage at the Manawatu River at SH2 (5.36 times median) and the Tokomaru River at Horseshoe Bend sites (16.53 times median).

Phormidium mats are likely removed by elevated river flows during flushing flows, which causes high shear stresses, abrasion by mobilised sediments and grinding action of tumbling gravel / cobble substrata (Clausen & Biggs, 1997, Biggs *et al.* 1999, Francoeur & Biggs 2006). Differences in these factors among sites may explain the variation in intensity of flow required to remove *Phormidium.* There was no notable difference among substrate composition at these sites (see Section 4.3 for further discussion). Data on suspended sediment, particularly during flushing flows, may assist in determining if this is a causative factor requiring further investigation.

As *Phormidium* mats form they go through a series of development stages; from initial attachment, to maturation and finally, dispersal. Dispersal occurs when bubbles of oxygen become entrapped within the mats causing them to detach from the substrate. Early stage mats are more firmly adhered to substrates and therefore would require more intense flushing to remove them. Future studies could include biomass assessments, for example using chlorophyll-a or dry mass collected from a known area, to investigate relationships between stage of growth, biomass and flushing flows.

4.2. Nutrients

Hydrological disturbances are critical in regulating *Phormidium* abundance. High energy events ‘re-set’ the system, however, this does not explain why some rivers / sites have blooms and others with similar hydrological conditions do not.

Additionally, the relationship among *Phormidium* and water column nutrients is complex due to the thick and cohesive structure of *Phormidium* mats.

Wood *et al*. (2014) used microelectrodes to show that a boundary layer (ca. 0.1 mm to 0.2 mm thick) exists between the mat surface and river water, which may restrict fluxes of nutrients from the river water into the mat. While the boundary layer may limit nutrient exchange, it facilitates the formation of geochemical conditions within mats that are very different to those of the surrounding water column. Wood *et al*. (2014) demonstrated that photosynthetic activity by *Phormidium* mats during daylight results in elevated pH (> 9) and super-saturated oxygen conditions (> 10 mg/L) within the mat environment. However, night-time respiration can result in oxygen depletion to less than 4 mg/L. They suggested that collectively these conditions might facilitate phosphorus release from the sediment trapped within and beneath *Phormidium* mats. This phosphorus is then available for growth and this may partially explain how *Phormidium* can form such high biomass in rivers with very low phosphorus. Wood *et al*. (2014) tested the interstitial *Phormidium* mat water and found that it had 300 times more DRP than bulk river water. Other elements such as iron were also significantly elevated. This creates a situation where the net diffusive nutrient flux through the boundary layer would be out of the mat. Therefore, it is highly unlikely that the mats are taking up any DRP from the river water and further research is required to investigate whether DIN concentration are also elevated within the mat water.

Under this scenario, once *Phormidium* mats are established, water column DRP concentrations are likely to be of little relevance to biomass accrual. This explains why no significant responses to increased nutrients were observed in this study, despite such relationships been reported previously for periphyton in New Zealand rivers (Biggs & Close 1989).

Low nutrient concentrations are required (Biggs 1990) during the initial stage of mat formation. It is likely that at this stage, water column nutrients define whether *Phormidium* will be dominant. Some cyanobacteria are known to be very adept at luxury uptake of phosphorus. They can store phosphorus in their cells for later cell division (Nausch *et al*. 2004). It is possible that *Phormidium* has the ability to do this. During the initial stage of mat formation this may give them a competitive advantage over other periphyton when bulk river water phosphorus concentrations are low. Wood and Young (2012) proposed a water column DRP threshold of less than 0.01 mg/L as favouring *Phormidium* blooms. In this study, three of the five sites confirmed this (Figure 16) and further assessment of data from 45 rivers in the Manawatu region also showed that sites with blooms, also generally had low water column DRP (Figure 16). There were two sites that experienced *Phormidium* blooms when the water column DRP was greater than 0.01 mg/L (Figure 21) and others were also identified during the analysis of the other 45 sites in the Manawatu region. However, DRP levels were still relatively low at ca. 0.02 mg/L. Another common feature of the sites in this study is that they are downstream of STPs. Both the Feilding and PNCC STPs use alum

dosing to reduce DRP concentrations during their treatment process. This reduces water column DRP, but results in an increase in the phosphorus load of discharged particulates (Hamill 2013). As stated above, *Phormidium* are adept at 'capturing' fine-grained extraneous particles, and the geochemical processes within the mats then facilitate phosphorus release. We speculate that this may explain the dominance of *Phormidium* at these sites despite the water column DRP being higher than the 0.01 mg/L suggested that favours *Phormidium* growth. Further investigation is required to confirm this and explore other possible reasons for the higher coverage of *Phormidium* below the STP. Further analysis of the larger Horizons monthly periphyton dataset may assist with this.

Molecular techniques have shown that cultures of *Phormidium* isolated from New Zealand rivers cannot fix nitrogen. This in concert with culture-based studies suggests that increased DIN concentrations (Heath *et al*. 2014a) may be required before *Phormidium* will bloom. Although the concentrations of nitrogen within the mat environment have not yet been investigated, it is likely that these are also elevated. *Phormidium* co-occurs in these mats with other microbial taxa, such as bacteria, diatoms and green algae. Brasell *et al*. (2014) used molecular techniques that demonstrated diverse bacterial communities among the mats. Preliminary data suggest that these include species capable of nitrogen fixation. Thus, although *Phormidium* itself cannot fix nitrogen, other organisms within the mat may provide sufficient quantities for growth once mats are established.

Wood and Young (2012) suggested a water column DIN threshold of greater than 0.10 mg/L promoted *Phormidium* blooms, and this has been collaborated by other studies (Heath *et al* 2013, Wood and Bridge 2014). In general this threshold holds true based on the findings of this study with four of the five sites that experience *Phormidium* blooms conforming to this model (Figure 21), and all sites (except the same one) in the analysis of the 45 samples from the Manawatu region. The DIN concentration at the Tokomaru River at Horseshoe Bend site are below this threshold, however, this site experiences prolonged *Phormidium* blooms. Reasons for this are unknown but may be related to the increased intensity in flow that is required to remove *Phormidium* mats at this site (see Section 4.1). This would provide longer period for mats to establish, or result in larger starting inoculums following flush events and these may already contain other nitrogen-fixing organisms. Because DRP and DIN concentrations are very low at this site, this likely prevents other algae out competing the *Phormidium*, and once established the mats may be able to source and recycle nitrogen internally. Further research would be required to confirm this.

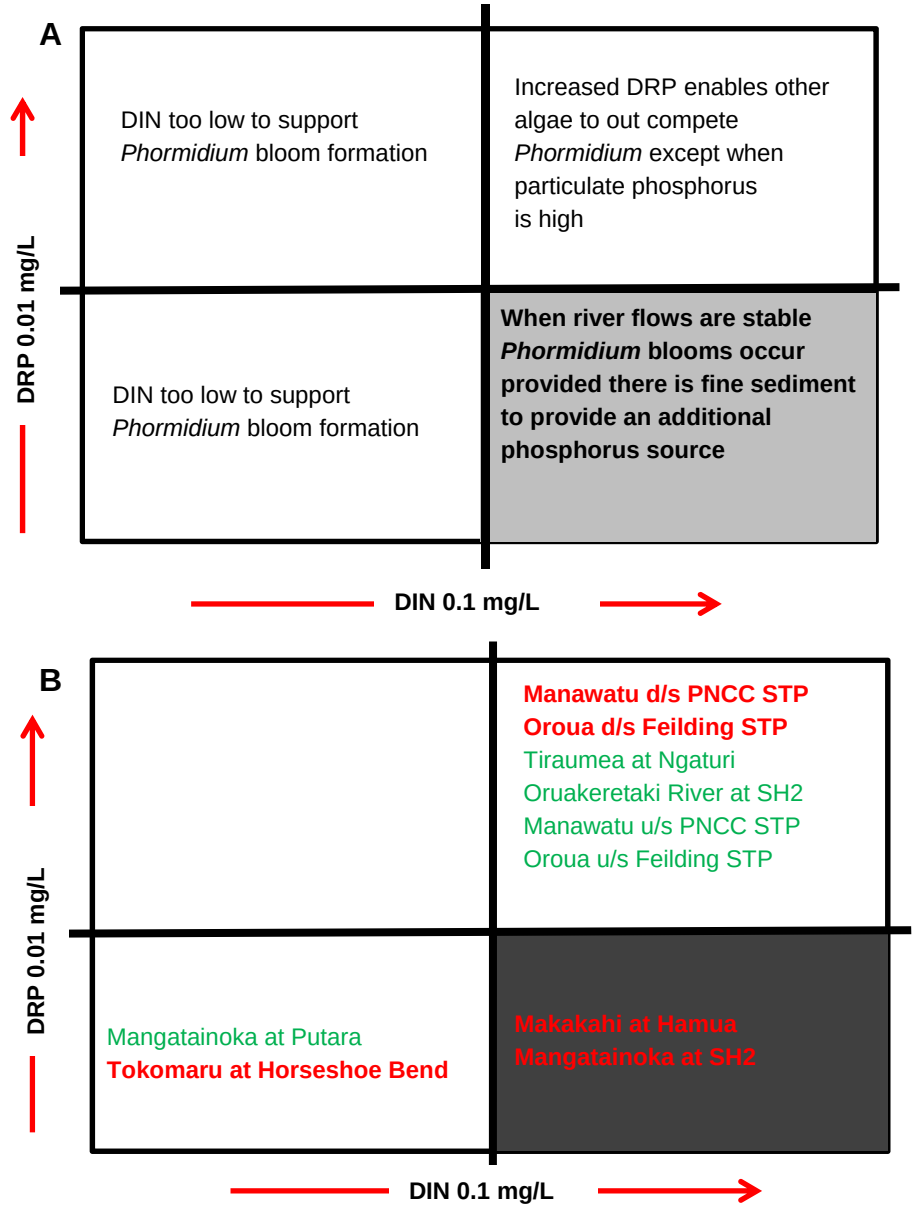

Figure 21. A) Proposed dissolved reactive phosphorus (DRP) and dissolved inorganic nitrogen (DIN) thresholds that promote *Phormidium* blooms when river flow is stable. B) Location of study sites within the *Phormidium* DRP/DIN threshold model. Sites in red experience *Phormidium* blooms (> 20% coverage). u/s = upstream, d/s = downstream, PNCC = Palmerston North City Council, STP = sewage treatment plant. SH2 = State Highway Two.

4.3. Substrate size

Although substrate stability is inherently linked with river flow, *i.e.* lower flows are required to move smaller sized substrate, there was no apparent relationship between *Phormidium* percentage coverage and substrate size. Sites with substrate of almost identical size (*i.e.* upstream/downstream of the STPs) had markedly different *Phormidium* coverage. Health *et al*. (2013) identified a slight positive relationship with

Phormidium coverage and increasing substrate size in a study in the Hutt River. They suggested this was due to:

1. Large, embedded substrates tend to remain stable in faster river flows compared with smaller unconsolidated substrates.
2. Larger substrate provide more 'refuges' during flushing flow events as they have a higher proportion of heterogeneity (*i.e.* cracks and crevices; Bergey 2005, Murdock & Dodds 2007) and these provide sites from which colonisation of the substrate can rapidly begin.

In this study the sites with the greatest portion of boulders had completely contrasting *Phormidium* coverage *i.e.* some of the highest *Phormidium* coverage was recorded at the Tokomaru River at Horseshoe Bend site, whilst almost no *Phormidium* was recorded at the Mangatainoka River at Putara site. So the actual size of substrate does not appear to be a critical factor in facilitating *Phormidium* blooms. However, at sites with *Phormidium* blooms, increase substrate stability will enable more prolonged blooms *i.e*. the Tokomaru River at Horseshoe Bend site.

During future studies it would be useful to measure turbidity or suspended solids to determine the amount of fine sediment in the water column. Wood *et al*. (2014) suggests that *Phormidium* 'captures' sediment from the water column that is then used as a source of phosphorus. Determining variation in the amount of fine sediment during *Phormidium* accrual may provide additional information on its role in bloom formation.

4.4. Toxins

Total anatoxin concentrations and the relative composition of structural variants showed high spatial and temporal variability among and within rivers. This result is consistent with data from a previous study of rivers in the Manawatu-Whanganui region (Wood &Young 2011) and other recent research in the Hutt River (Wellington), Maitai River (Nelson) and in five Southland rivers (Heath & Wood 2010, Heath *et al*. 2011, Wood & Bridge 2014).

There was a marked increase in total toxin concentrations at several sites and on multiple occasions within a 1 to 2 week period. There are several possible explanations for this. The most likely reason is that toxic and non-toxic genotypes are co-occurring in the *Phormidium* mats (Heath *et al*. 2010, Wood *et al*. 2010). The relative amount of each genotype will affect the total toxin in each sample. Wood *et al*. (2012) isolated multiple single filaments from a 1 cm^2 area of mat and grew each into uni-cyanobacterial cultures. Molecular and chemical analysis were then used to show that both toxic and non-toxic genotypes co-exist, and that among toxic strains the

concentration of toxin produced can vary 100-fold. Consequently, not only the relative amount of toxic versus non-toxic genotypes is important, but also the abundance of toxic genotypes that produce higher anatoxin quotas (*i.e.* the amount of toxin produced per cell).

Variables that regulate the presence and abundance of each genotype within a mat are unknown. Heath *et al*. (2010) suggested that the toxin-producing strains in the Hutt River 'out-competed' non-toxic *Phormidium* strains at temperatures above 15˚C. In this study, toxins were detected in a wide range of temperatures, suggesting that temperature is unlikely to regulate the abundance of toxic and non-toxic strains.

A second hypothesis is that the chemical conditions within the mat (*e.g*. low oxygen, elevated pH) or the surrounding water caused an up-regulation in the amount of toxin produced per cell. In a culture-based study Heath *et al*. (2014b) showed increased nitrogen and phosphorus concentrations resulted in higher anatoxin quotas. In this study no correlations were identified between toxin concentration and DIN or DRP. However, Wood *et al*. (2014) demonstrated that the chemical and nutrient conditions within a *Phormidium* mat are significantly different from the surrounding water column. The majority of the cells within a mat would be exposed to the 'within mat' environment, so there needs to be studies correlating toxin production to these conditions to further explore any relationships between elements / nutrients and toxin production.

The mats are also collected at different growth stages and this may account for the variable toxin concentrations. Heath *et al*. (2014b) and Harland *et al*. (2013) demonstrated that toxin quota peaked in the initial growth phase. This scenario seems less likely because most of the high toxin values came from reasonably well-developed mats.

Over the study period there was no correlation between toxin concentrations and mean *Phormidium* coverage. A similar result has been shown in other recent studies (Wood *et al*. 2010, Heath & Wood 2010, Heath *et al*. 2011). The majority of the toxin concentrations in this study were relatively low compared to those from a survey of seven rivers throughout New Zealand (Wood *et al*. 2010). Wood *et al*. (2010) measured a maximum total anatoxin concentration of 12.8 mg/kg FDW. The large majority of positive samples in this study were less than 5 mg/kg FDW. The generally lower results in this study may be because 10 samples were pooled at each site. For example, if five samples from a site contained no toxins, the pooled average would be reduced. In Wood *et al*. (2010) all samples were analysed individually. The sample from the Makakahi River at Hamua site on 17 February 2012 had total toxin concentrations markedly higher (82.4 mg/kg FDW) than those measured in the Manawatu-Whanganui region previously or in the Wood *et al*. (2010) study. Similar or higher concentrations have been measured in the Hutt River (Heath *et al*. 2011).

Toxins were consistently absent from the Oroua River sites in this and the previous study (Wood & Young 2011), and at the Manawatu River upstream of PNCC STP site. This suggests the absence of toxic genotypes at these locations but molecular techniques would be required to confirm this assumption. The putative gene cluster involved in ATX production was recently characterised (Méjean *et al*. 2009), which has enabled molecular tools to be used to study the abundance of toxic and non-toxic genotypes.

Phormidium in New Zealand is known to produce ATX (Heath *et al*. 2010). However, no ATX was detected in *Phormidium* in this study and the previous work done in the Manawatu-Whanganui region. Similarly, ATX was not detected in samples from seven New Zealand rivers (Wood *et al*. 2010). Anatoxin-a degrades readily, especially in sunlight and at high pH; whereas the dihydro-compounds are more stable (Smith & Lewis 1987). This may partially explain the absence of ATX, although HTX is expected to be just as unstable as ATX. A more likely explanation is that the strains of *Phormidium,* present in the Manawatu-Whanganui region, do not produce ATX. Variability in the production of anatoxin variants has been demonstrated in New Zealand *Phormidium* strains previously (Wood *et al*. 2012). Among and within sites there was variability in the relative portion of each anatoxin variant produced. Heath *et al*. (2014b), suggest that strains that produce more HTX, or that the up-regulation of HTX occurs in phosphorus-replete conditions. An initial analysis of this data does not indicate this scenario as the sites with the lowest HTX are also the sites with the least DRP.

4.5. Conclusions

The key findings of this study are:

- The flushing flow that results in 90% of records being below 20% *Phormidium* coverage, varied markedly between sites. If flushing flows are used to inform when *Phormidium* might be present at a site, or when monitoring is required, then these need to be site-specific.
- The thresholds in water column DRP and DIN concentrations that rendered a river more likely to experience *Phormidium* blooms in the Manawatu-Whanganui region were less than 0.01 mg/L and greater than 0.2 mg/L respectively. The exceptions to this where; sites downstream of sewage treatment plants were the DRP was ca. 0.02 mg/L, and the Tokomaru River at Horseshoe Bend site were DIN was very low (less than ca. 0.1 mg/L). Further investigation is required at these sites.
- There were no correlations between anatoxin production (and variants produced) and *Phormidium* coverage, or any of the physico-chemical variants measured. We suggest the variability in toxin concentration is largely due to differences in the relative abundance of toxic and non-toxic genotypes within each mat.

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7. APPENDICES

Appendix 1. Long-term median flows for sampling sites. Refer to Figure 1 for location of sites. * estimated from available data. No long-term statistics were available for this site.

Site	Long-term median flow ($m^3 s^{-1}$)
Makakahi River at Hamua	3.18
Mangatainoka River at Larsons Rd	2.13
Mangatainoka River at Pahiatua Town Bridge	8.90
Oroua River at Almdale Slackline	7.10
Oruakeretaki River at State Highway Two	1.42*
Tiraumea River at Ngaturi	7.21
Tokomaru River at Riverland Farm	1.25
Manawatu River at Teachers College	73.4

Appendix 2. Site names for data given in Figure 18. Note: SH2 = State Highway Two, u/s = up stream, d/s = downstream, PNCC = Palmerston North City Council, STP = sewage treatment plant, DOC = Department of Conservation.

Site no.	Site name	Site no.	Site name
1	Mangawhero River at DoC Headquarters	31	Oroua River at Awahuri Bridge
2	Mangawhero River d/s Ohakune STP	32	Rangitikei River at McKelvies
3	Mangawhero River u/s Ohakune STP	34	Manawatu River at Upper Gorge
4	Makotuku River at Raetihi	35	Mangapapa River at Troup Rd Bridge
5	Mangawhero River at Pakihi Rd Bridge	36	Mangaatua River d/s Woodville STP
6	Makotuku River u/s Raetihi STP	37	Mangaatua River u/s Woodville STP
7	Makotuku River d/s Raetihi STP	38	Manawatu River at Hopelands
8	Rangitikei River at Pukeokahu	39	Manawatu River at Teachers College
12	Rangitikei River at Mangaweka	40	Manawatu River u/s PNCC STP
13	Oroua River at Apiti Gorge Bridge	41	Manawatu River d/s PNCC STP
15	Pohangina River at Piripiri	42	Mangatainoka River d/s DB Breweries
17	Rangitikei River at Onepuhi	43	Mangatainoka River at SH2
18	Tamaki River at Reserve	44	Manawatu River at Opiki Bridge
19	Oroua River at Almadale Slackline	46	Mangatainoka River d/s Pahiatua STP
20	Pohangina River at Mais Reach	47	Mangatainoka River u/s Pahiatua STP
21	Kumeti River at Te Rehunga	49	Tiraumea River at Ngaturi
22	Mangatera River u/s Dannevirke STP	50	Tokomaru River at Horseshoe bend
23	Mangatera River d/s Dannevirke STP	51	Makuri River at Tuscan Hills
25	Manawatu River at Weber Rd	52	Makakahi River at Hamua
26	Tamaki River at Stephensons	54	Ohau River at Gladstone Reserve
28	Oroua River u/s Feilding STP	56	Mangatainoka River at Putara
29	Oroua River d/s Feilding STP	57	Waikawa River at Nth Manakau Road
30	Oruakeretaki River at SH2		

Appendix 3. Toxin data (mg/kg freeze-dried weight) for each sample. Note: ATX = anatoxin-a, HTX = homoanatoxin-a, dhATX = dihydro-anatoxin-a, dhHTX = dihydro-homoanatoxin-a. SH2 = State Highway 2, u/s = up stream, d/s = downstream, PNCC = Palmerston North City Council, STP = sewage treatment plant.

Date	ATX	HTX	dhATX	dhHTX	Total
Makakahi at Hamua					
6/01/12	0.00	0.03	0.06	0.00	0.09
13/01/12	0.00	0.02	0.02	0.00	0.04
20/01/12	0.00	0.09	0.16	0.00	0.24
27/01/12	0.00	0.37	2.85	1.17	4.38
3/02/12	0.00	1.04	6.78	3.97	11.79
10/02/12	0.00	0.20	0.85	0.15	1.19
17/02/12	0.00	33.16	4.40	44.82	82.38
16/03/12	0.00	0.70	0.35	0.18	1.24
30/03/12	0.00	0.05	0.00	0.00	0.05
4/04/12	0.00	0.03	0.02	0.00	0.05
13/04/12	0.00	0.21	0.02	0.00	0.23
20/04/12	0.00	0.45	0.22	0.23	0.90
28/04/12	0.00	0.09	0.01	0.00	0.10
4/05/12	0.00	0.81	0.54	0.66	2.00
31/08/12	0.00	0.00	0.00	0.00	0.00
7/09/12	0.00	0.00	0.00	0.00	0.00
12/10/12	0.00	0.00	0.00	0.00	0.00
2/11/12	0.00	0.00	0.00	0.00	0.00
9/11/12	0.00	0.00	0.00	0.00	0.00
16/11/12	0.00	0.01	0.03	0.00	0.04
23/11/12	0.00	0.00	0.01	0.00	0.01
30/11/12	0.00	0.01	0.05	0.00	0.06
7/12/12	0.00	0.07	0.94	0.05	1.05
14/12/12	0.00	0.00	0.02	0.00	0.03
21/12/12	0.00	0.37	0.27	0.38	1.02
28/12/12	0.00	0.06	0.11	0.02	0.19
4/01/13	0.00	0.00	0.03	0.00	0.03
11/01/13	0.00	0.05	0.17	0.02	0.24
25/01/13	0.00	0.12	0.40	0.17	0.69
17/05/13	0.00	0.02	0.00	0.00	0.02
24/05/13	0.00	0.00	0.00	0.00	0.00
Manawatu d/s PNCC STP					
26/09/12	0.00	0.00	0.00	0.00	0.00
14/11/12	0.00	0.00	0.00	0.00	0.00
21/11/12	0.00	0.00	0.00	0.00	0.00
28/11/12	0.00	0.00	0.00	0.00	0.00
5/12/12	0.00	0.00	0.00	0.00	0.00
12/12/12	0.00	0.00	0.00	0.00	0.00
9/01/13	0.00	0.00	0.00	0.00	0.00
16/01/13	0.00	0.00	0.00	0.00	0.00
23/01/13	0.00	0.01	0.00	0.00	0.01
30/01/13	0.00	0.02	0.00	0.00	0.02
13/02/13	0.00	0.02	0.00	0.00	0.02
20/02/13	0.00	0.18	0.00	0.01	0.20
27/02/13	0.00	0.10	0.00	0.00	0.10
6/03/13	0.00	0.16	0.00	0.01	0.17
14/03/13	0.00	0.32	0.00	0.02	0.35
27/03/13	0.00	0.00	0.00	0.00	0.00
3/04/13	0.00	0.06	0.00	0.00	0.06
10/04/13	0.00	0.00	0.00	0.00	0.00
15/05/13	0.00	0.00	0.00	0.00	0.00
22/05/13	0.00	0.22	0.05	0.01	0.27

Date	ATX	HTX	dhATX	dhHTX	Total
Mangatainoka at SH2					
6/01/12	0.00	0.00	0.05	0.00	0.05
13/01/12	0.00	0.12	0.38	0.08	0.58
20/01/12	0.00	0.19	0.26	0.03	0.47
27/01/12	0.00	0.12	0.40	0.05	0.57
3/02/12	0.00	0.50	1.61	0.56	2.68
10/02/12	0.00	0.77	5.19	4.31	10.27
17/02/12	0.00	1.01	8.04	7.57	16.61
24/02/12	0.00	0.00	0.00	0.00	0.00
2/03/12	0.00	1.31	1.39	5.11	7.81
9/03/12	0.00	0.00	0.00	0.00	0.00
16/03/12	0.00	0.00	0.00	0.00	0.00
23/03/12	0.00	0.00	0.00	0.00	0.00
30/03/12	0.00	0.00	0.00	0.00	0.00
4/04/12	0.00	0.03	0.01	0.00	0.05
13/04/12	0.00	0.27	0.13	0.08	0.48
20/04/12	0.00	0.53	0.11	0.17	0.81
28/04/12	0.00	0.06	0.03	0.01	0.10
4/05/12	0.00	0.04	0.04	0.00	0.08
11/05/12	0.00	0.05	0.05	0.01	0.11
25/05/12	0.00	0.09	0.03	0.01	0.13
1/06/12	0.00	0.08	0.03	0.00	0.11
15/06/12	0.00	0.07	0.01	0.00	0.08
9/11/12	0.00	0.00	0.02	0.00	0.02
16/11/12	0.00	0.03	0.11	0.01	0.14
23/11/12	0.00	0.09	0.77	0.08	0.95
7/12/12	0.00	0.51	0.46	0.33	1.30
14/12/12	0.00	0.37	0.39	0.35	1.11
21/12/12	0.00	0.33	0.27	0.35	0.95
28/12/12	0.00	1.01	0.69	1.11	2.81
4/01/13	0.00	0.31	0.20	0.25	0.76
11/01/13	0.00	0.45	0.55	0.82	1.82
18/01/13	0.00	0.95	1.40	2.06	4.41
25/01/13	0.00	0.71	3.60	5.81	10.11
1/02/13	0.00	0.29	1.52	1.35	3.16
8/02/13	0.00	0.16	0.43	0.27	0.86
15/02/13	0.00	0.02	0.05	0.01	0.07
22/02/13	0.00	0.04	0.07	0.01	0.12
1/03/13	0.00	0.10	0.43	0.21	0.74
8/03/13	0.00	0.04	0.13	0.02	0.19
15/03/13	0.00	0.00	0.07	0.00	0.07
22/03/13	0.00	0.00	0.00	0.00	0.00
27/03/13	0.00	0.07	0.08	0.05	0.20
5/04/13	0.00	0.15	0.89	0.54	1.58
12/04/13	0.00	0.00	0.08	0.00	0.08
10/05/13	0.00	0.00	0.00	0.00	0.00
17/05/13	0.00	0.00	0.00	0.00	0.00
24/05/13	0.00	0.00	0.02	0.00	0.02

Appendix 3. *continued*

Date	ATX	HTX	dhATX	dhHTX	Total
Oruakeretaki at SH2					
12/10/12	0.00	0.00	0.00	0.00	0.00
16/11/12	0.00	0.01	0.03	0.00	0.04
30/11/12	0.00	0.02	0.04	0.00	0.06
25/01/13	0.00	0.07	0.02	0.00	0.09
1/02/13	0.00	0.02	0.05	0.01	0.08
8/02/13	0.00	0.09	0.02	0.01	0.12
15/02/13	0.00	0.15	0.03	0.01	0.18
22/02/13	0.00	0.17	0.04	0.01	0.21
1/03/13	0.00	0.03	0.02	0.00	0.05
8/03/13	0.00	0.22	0.11	0.03	0.35
15/03/13	0.00	0.49	0.23	0.17	0.90
12/04/13	0.00	0.08	0.01	0.00	0.09
4/05/13	0.00	0.13	0.02	0.01	0.16
10/05/13	0.00	0.15	0.02	0.00	0.18
17/05/13	0.00	0.16	0.02	0.00	0.18
24/05/13	0.00	0.25	0.02	0.01	0.27
Tokomaru at Horseshoe Bend					
6/01/12	0.00	0.02	0.00	0.00	0.02
13/01/12	0.00	0.21	0.01	0.00	0.22
20/01/12	0.00	0.00	0.04	0.00	0.04
3/02/12	0.00	0.90	0.25	0.23	1.38
10/02/12	0.00	1.93	1.23	2.08	5.24
30/03/12	0.00	0.06	0.03	0.00	0.09
4/04/12	0.00	0.09	0.02	0.00	0.11
13/04/12	0.00	0.06	0.01	0.00	0.07
20/04/12	0.00	0.06	0.04	0.00	0.10
28/04/12	0.00	1.90	0.34	0.37	2.61
4/05/12	0.00	0.38	0.07	0.03	0.48
11/05/12	0.00	2.05	2.87	1.29	6.21
25/05/12	0.00	0.46	0.23	0.24	0.92
1/06/12	0.00	0.26	0.41	0.11	0.78
8/06/12	0.00	0.17	0.07	0.01	0.24
15/06/12	0.00	0.15	0.04	0.01	0.20
28/11/12	0.00	0.02	0.00	0.00	0.02
28/12/12	0.00	0.00	0.00	0.00	0.00
9/01/13	0.00	0.00	0.00	0.00	0.00
23/01/13	0.00	0.02	0.00	0.00	0.02
30/01/13	0.00	0.16	0.02	0.00	0.18
7/02/13	0.00	0.26	0.03	0.00	0.30
13/02/13	0.00	0.00	0.00	0.00	0.00
20/02/13	0.00	0.42	0.06	0.01	0.50
27/02/13	0.00	0.31	0.07	0.03	0.41
8/05/13	0.00	0.18	0.07	0.01	0.26
15/05/13	0.00	0.20	0.01	0.00	0.21
22/05/13	0.00	0.17	0.04	0.01	0.22
5/06/13	0.00	0.14	0.05	0.02	0.20

Manawatu u/s PNCC STP					
12/12/12	0.00	0.00	0.00	0.00	0.00
9/01/13	0.00	0.00	0.00	0.00	0.00
Tiraumea at Ngaturi					
14/12/12	0.00	0.00	0.00	0.00	0.00
21/12/12	0.00	0.00	0.00	0.00	0.00
4/01/13	0.00	0.00	0.00	0.00	0.00
11/01/13	0.00	0.01	0.03	0.02	0.07
18/01/13	0.00	0.00	0.00	0.00	0.00
25/01/13	0.00	0.00	0.00	0.00	0.00
1/02/13	0.00	0.00	0.00	0.00	0.00
15/02/13	0.00	0.06	0.00	0.01	0.07
22/02/13	0.00	0.03	0.00	0.00	0.03
1/03/13	0.00	0.03	0.00	0.01	0.05
8/03/13	0.00	0.05	0.00	0.02	0.07
15/03/13	0.00	0.06	0.00	0.02	0.08
22/03/13	0.00	0.12	0.00	0.04	0.16
27/03/13	0.00	0.13	0.00	0.03	0.16
12/04/13	0.00	0.06	0.00	0.00	0.06
19/04/13	0.00	0.12	0.00	0.00	0.12
Oroua u/s Feilding STP					
10/02/12	0.00	0.00	0.00	0.00	0.00
17/02/12	0.00	0.00	0.00	0.00	0.00
24/02/12	0.00	0.00	0.00	0.00	0.00
8/06/12	0.00	0.00	0.00	0.00	0.00
28/12/12	0.00	0.00	0.00	0.00	0.00
3/01/13	0.00	0.00	0.00	0.00	0.00
9/01/13	0.00	0.00	0.00	0.00	0.00
16/01/13	0.00	0.00	0.00	0.00	0.00
Oroua d/s Feilding STP					
20/12/12	0.00	0.00	0.00	0.00	0.00
28/12/12	0.00	0.00	0.00	0.00	0.00
3/01/13	0.00	0.00	0.00	0.00	0.00
9/01/13	0.00	0.00	0.00	0.00	0.00
16/01/13	0.00	0.00	0.00	0.00	0.00
23/01/13	0.00	0.00	0.00	0.00	0.00
30/01/13	0.00	0.00	0.00	0.00	0.00
16/04/13	0.00	0.00	0.00	0.00	0.00
1/05/13	0.00	0.00	0.00	0.00	0.00
15/05/13	0.00	0.00	0.00	0.00	0.00
5/06/13	0.00	0.00	0.00	0.00	0.00